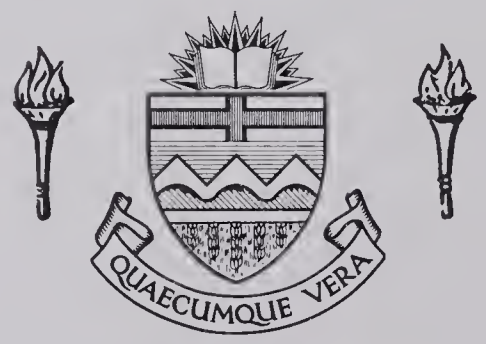

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A QUANTITATIVE STUDY ON THE INTRACUTANEOUS ABSORPTION OF A C^{14}-LABELED COMPOUND

by

JOHN NICHOLAS HLYNKA, B.Sc., M.Sc.

A Thesis submitted to the Faculty of Graduate Studies in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

FACULTY OF PHARMACY

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled:

"A QUANTITATIVE STUDY ON THE INTRACUTANEOUS ABSORPTION OF A C^{14}-LABELED COMPOUND"

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To my wife Sheila, whose patience, understanding and encouragement contributed immeasurably to the realization of this dissertation.

ABSTRACT

A method using the skin of guinea pigs has been developed for quantitatively measuring the transepidermal, intracutaneous absorption of radioisotope-labeled compounds. The intracutaneous absorption of topically applied C^{14}-labeled salicylic acid was measured by the described method and compared to the systemic absorption of the drug as determined by plasma analysis. The comparisons are based on studies of absorption as a function of rest time and of concentration.

Systemic absorption through the skin was found to be not representative of intracutaneous absorption in either study. These results indicate that percutaneous absorption measurements should be made for both the intracutaneous and systemic phases. The effects of the rest time and concentration variables on intracutaneous absorption were in good agreement with effects predicted theoretically. This situation did not hold true for the systemic values obtained. Possible explanations for the differences between the intracutaneous and systemic measurement results and between the experimental and theoretical data are presented.

ACKNOWLEDGEMENTS

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INTRODUCTION

The percutaneous absorption of a drug from a topically applied vehicle may be considered as being basically a two-phase process where the drug penetrates from the surface of the skin into the living epidermal and dermal tissues and then into the circulatory system of the body. Absorption by each of these two phases will be more specifically referred to in this dissertation as intracutaneous absorption and systemic absorption respectively while reference to absorption by both phases simultaneously will be represented by the more general term of percutaneous absorption. The degree of absorption intended in each of these phases may vary depending upon the reason for initiating percutaneous absorption. For example, when localized dermal therapy is necessary a maximum amount of drug should be concentrated in the intracutaneous phase at the required site. Subsequent systemic absorption should be kept to a minimum, however, in order to prolong the contact of the drug with the skin tissues and to reduce the possibility of the drug causing undesirable systemic side-effects. Therefore studies on percutaneous absorption should be directed to the individual investigation of absorption in each of these phases.

The interest of pharmaceutical formulators in percutaneous absorption derives from the many claims that formulation factors can influence the rate and the extent of the absorption process. If such is the case the objective of promoting better skin therapy should be to design a preparation for a given

drug which would afford maximum release of the drug to the surface of the skin, maximum intracutaneous absorption and minimum systemic absorption. The formulation factors which influence drug absorption by each phase must be identified, however, before such a goal can be realized. Unfortunately, these factors and the magnitude of their effects have not been completely elucidated as evidenced by the contradictory results too frequently reported by investigators in this field.

Some of this controversy probably can be attributed to the many methods which have been used to measure percutaneous absorption, most of which must be considered as inadequate. This is particularly true for measurements of intracutaneous absorption. The methods used to measure absorption by this phase have resulted in either poor reproducibility or in only a qualitative estimation of the amount of drug absorbed. To properly evaluate the relative effects which formulation factors may have upon intracutaneous absorption, a quantitative method of measurement is required which would be sensitive enough to detect any changes in absorption caused by these factors. An alternative to measuring intracutaneous absorption would be to extrapolate absorption results from systemic measurements to represent absorption by the skin tissues as well. Such extrapolation would be valid only if absorption by the two phases were proportional and only if the formulation factor being studied influenced absorption in both phases to the same degree. Therefore, the validity of the alternative would still depend primarily on the availability of a quantitative method for

measuring intracutaneous absorption.

A theoretical consideration of the skin barriers to drug penetration and of the mechanisms by which a drug may overcome these barriers indicates that the rate and extent of drug absorption by the systemic phase may not adequately measure absorption by the intracutaneous phase. Similarly, a survey of the methods used to measure percutaneous absorption indicates that an adequate method providing quantitative, reproducible measurements of intracutaneous absorption has not yet been developed.

LITERATURE SURVEY

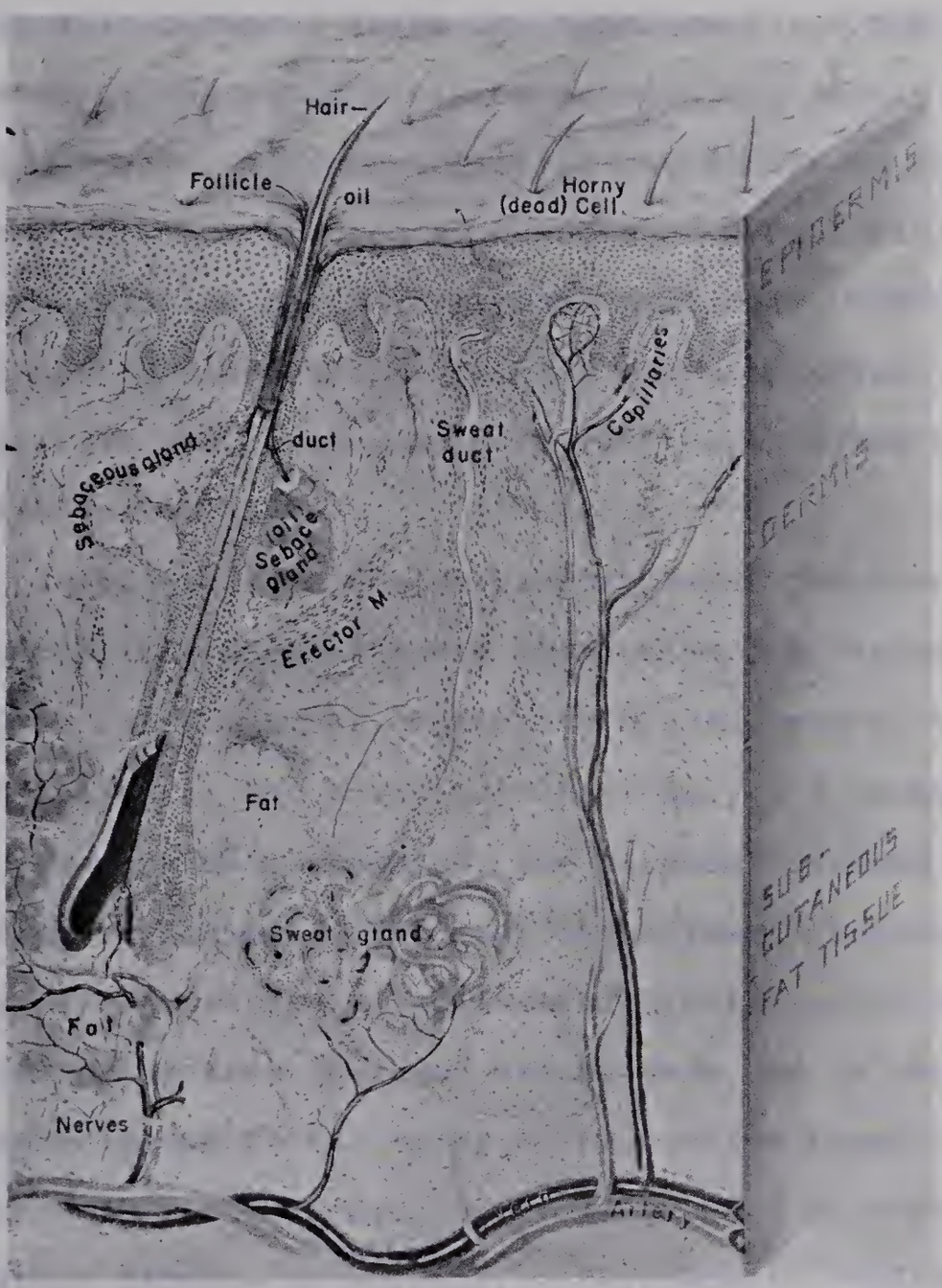
A. Factors Encountered in Percutaneous Absorption

One of the primary functions of the skin is that of protection (1,2,3). It serves this function well by preventing the undesirable loss of body fluids to the environment while at the same time resisting the entry into the body of harmful light rays, microorganisms and physical and chemical agents from the external environment. The defence mechanisms of the skin against foreign substances become of particular interest in dermatological therapy since the barriers to penetration of toxic chemical molecules are similarly effective against the penetration of drug molecules intended for beneficial therapy. An understanding of the nature of such barriers therefore becomes essential to the formulator of dermatological preparations. Attempts must be made to modify or by-pass these barriers with the formulation so that a maximum concentration of drug can be promoted within the skin without significantly disrupting the normal defensive mechanisms.

Anatomy

The skin may be considered as consisting of two basic layers, the epidermis and the dermis (3,4) (Figure 1). Although they are physically joined by a membrane at the dermatological junction the anatomical characteristics of these two layers differ significantly as might be expected since they have different embryonic origins (3,4). The epidermis which is much thinner than the dermis at maturity is derived from the embryonic ectoderm while the dermis

Figure 1. Anatomical zones encountered in percutaneous absorption (from Griesemer, R.D., Soc. Cosmetic Chemists, 11, 80 (1960)).


Hair
Follicle
oil
Horny
(dead) Cell
EPIDERMIS
Capillaries
Sweat
duct
duct
Sebaceous gland
Hair
Sebac
gland
Erector M
DERMIS
Fat
Sweat gland
SUB-
CUTANEOUS
FAT TISSUE
Fat
Nerves

originates in the mesoderm. The combined thickness of the two varies from about 0.5 mm to approximately 4 mm depending largely on which part of the body they are located (4,5). These two segments rest upon subcutaneous fat which is occasionally referred to as the hypoderm (3). There is no membrane delineating a division between the dermis and the fat tissue (6). The connection of the dermis to the subcutaneous fat is effected by bundles of dermal fibers extending down into the fat in irregular patterns. Subcutaneous fat is not considered as a part of the skin proper (4). Basically it serves as a support for the skin and anchors the skin to the body.

The epidermis is the major portion of the skin involved in protection against entry by foreign substances (7). It may be classified as consisting of five layers, the stratum corneum which is the horny surface layer, the stratum lucidum, the stratum granulosum, the stratum spinosum and the stratum germinativum which is located immediately above the dermis (8). Just as the epidermis is believed to be the external protective division of the skin, so is the stratum corneum accepted as the external protective layer of the epidermis (5). It is made up of lipids and dead cells consisting largely of keratin, a hygroscopic, sulfhydryl-containing protein (1). This combination presents a rugged shield against foreign invaders. The dead cells and lipids are regularly sloughed off as the intercellular cement connecting the cells disintegrates. To maintain this

defensive layer, therefore, new cells must be produced to replace those lost. Since the cells of the stratum corneum are dead they cannot regenerate themselves and must be replaced from epidermal layers below. Cells of epidermis develop and multiply in the deepest of the epidermal layers, the stratum germinativum (9, 10). As multiplication in this region continues the cells are gradually pushed up into succeeding layers where the process of keratinization progressively develops until dead keratin cells appear in the stratum corneum. With the exception of the palms of the hands and the soles of the feet, the epidermis is approximately 60 to 100 microns thick on most parts of the body. In these areas the stratum corneum itself may reach a thickness of as much as 600 microns (5). The surface of the skin is interrupted by hair follicles and sweat gland ducts. A lipid surface film, composed of sebum, sweat and desquamating stratum corneum, is weakly acidic in nature and is therefore referred to as the "acid mantle" of the skin (11). This mantle has been claimed to have bacteriostatic characteristics, probably due to the fatty acids in the sweat and sebum on the surface of the skin (12).

Unlike the differentiating cell layers of the epidermis the dermis is made up chiefly of inert fibrous tissues. Collagen fibers, elastin fibers and relatively few living cells are packed together in a ground substance consisting primarily of mucopolysaccharides (6). The dermis is regarded as having two layers with no clearly defined

separation. The upper portion of the dermis projects into the epidermis in cone-like papillae. This region is known as the papillary layer while the region directly below is referred to as the reticular layer because of the denser network of fibers present (13). The fibrous nature of the dermis lends to it a flexibility which, along with the subcutaneous fat, provides support for the epidermis and also permits lateral movement for the skin. Hair follicles are developed by the invagination of the epidermis into various depths of the dermis or subcutaneous fat (14). A matrix of cells forms at the base of the follicle which gives rise to the hair bulb and eventually the hair shaft. Although the hair follicle extends into the dermis and occasionally as deep as the fat tissue it should be remembered that the continuous external sheath around the follicle is of epidermal origin and nature. This epidermal sheath becomes thinner around the deeper follicular regions and gradually loses some of its epidermal characteristics. The keratinized stratum corneum in particular disappears according to Odland (15), approximately 200 microns below the surface of the skin. Located adjacent to and continuous with many hair follicles are sebaceous glands. Like hair follicles they are also contained within an epidermal sheath. These glands produce sebum which lubricates the hair follicle and is present in the lipid film on the surface of the epidermis. Sebum is a lipid substance composed chiefly of free fatty acids and glycerides (16, 17). Sebaceous glands

are not distributed uniformly over the body and their activity differs according to the body distribution. Another skin appendage that must be considered is the sweat gland. The eccrine sweat glands are of more interest in percutaneous absorption than are the larger apocrine glands because they are located throughout most of the skin area. The apocrine glands are found only in regions such as the axilla, the external auditory meatus, the eyelids, the anogenital surfaces and the nipple of the breast (18). The coiled main body of the eccrine sweat gland is located in the dermis. A duct extends upward from this coil through the skin and opens onto the surface. The aqueous secretions from these glands make up a major portion of the "acid mantle" (12).

The circulatory system of the skin is primarily involved in the second phase of percutaneous absorption. Blood is supplied to the skin from a major network, the rete cutaneum, located in the subcutaneous tissue (19). Vascular branches passing upward from this network pass through the dermis and around the various appendages. Capillary beds surround the bulbs of the hair follicles and the sebaceous and sweat glands. They also form a network immediately below the epidermis to supply the cells of the stratum granulosum. The main body of the dermis is supplied only very sparingly with capillaries (19). The lymph circulation runs parallel to the blood supply. It originates in the papillar, glandular and follicular regions and joins the venous system near the heart (20). It has

been proposed that drug molecules whose molecular size is too large for passage through the blood vessel walls are carried away systemically by the lymphatics (1).

2. Barrier Characteristics

Although there is general agreement among investigators regarding the effectiveness of the barriers of the skin to percutaneous absorption, there are diversified opinions as to the exact location of such barriers and the mechanisms by which they function. Lipoidal, electrical, physical and aqueous barriers have all been proposed to exist throughout the skin.

The lipid film on the surface of the skin and lipids in the superficial tissues of the epidermis might be expected to combine in forming a lipoidal barrier. Depending on the effectiveness of such a barrier the absorption of lyophilic molecules would be enhanced through this region while the absorption of hydrophilic molecules would be resisted. It is believed that the lipids in the epidermis do play an important role in this respect (1,2,21). However, as both Rothman (22) and Malkinson (23) have pointed out, the importance of the surface film as a lipoidal barrier is probably over-emphasized. The continuous sloughing off of keratin and the presence of emulsifiers such as cholesterol in the sebum likely result in the surface film being a discontinuous film rather than an occlusive barrier.

The "major barrier" to percutaneous absorption is

believed to exist between the surface film and the stratum granulosum. This barrier was first reported by Rein (24) who depicted it as a zone between the stratum corneum and stratum granulosum. Other workers (25,26,27) concurring with this concept narrowed its location to the base of the stratum corneum. This philosophy has led to the stratum lucidum being occasionally referred to as the "barrier zone" (28). Studies by Monash (29), Monash and Blank (30), and Lawler, Davis and Griffiths (31), however, indicate that the complete stratum corneum may function as the barrier. The proposed mechanisms by which this area serves as a barrier also vary. Rein (24) originated the concept of the barrier being electronegative, thereby attracting cations and repelling anions. Tregear (2) on the otherhand conceived the resistance of the barrier to be a physical property of the dead, keratinized cells in the stratum corneum. The effectiveness of this barrier zone appears to vary over different parts of the body. For example, Smith (32) reported that some compounds penetrate through scrotal skin more readily than through abdominal skin in humans. It has been proposed that drugs which enter the follicular canals and sebaceous glands penetrate into the skin more readily than those which penetrate only from the surface (33,34,35). The absence of stratum corneum in part of the epidermal sheath surrounding the walls of these appendages (15) might preclude the presence of a "major barrier" like the one which exists on the surface of the epidermis. The

work of Tregear (36), however, showed that on pigs the hair follicle was no more penetrable than an equivalent area of epidermis on the living skin.

Once the penetrating molecule passes through the lipoidal and "major" barriers it would next encounter the living epidermal and dermal layers respectively. This region has been referred to as the aqueous barrier due to the increased water content of the tissues here as compared to that in the stratum corneum (37). On this basis, the absorption of molecules which have little affinity for water would be expected to be retarded by this area. Hydrophilic compounds on the otherhand would be expected to readily diffuse through these tissues and into the blood stream.

From the work reported it would appear that a drug penetrating into and through the skin may be subjected in turn to a lipoidal barrier, a "major barrier" zone and an aqueous barrier. In healthy skin, the effectiveness of each respective barrier in resisting the penetration of a given drug probably will be determined by the physical and chemical properties of the drug molecule (1).

B. Mechanisms of Percutaneous Absorption

The mechanism of the percutaneous absorption of a drug may be interpreted as the processes involved in effecting the penetration of a drug into the intracutaneous and systemic phases. Although these processes have not been clearly defined (34,38) there appears to be general agreement that drugs are absorbed

percutaneously by passive diffusion (2,37) and that the degree of absorption is primarily a function of the combined properties of the drug preparation and of the skin (1,37,39). Of major importance in the present study is whether the processes responsible for intracutaneous and systemic absorption are the same. If they should prove to be different then the relative absorption by the two phases might be expected to differ as well. This situation would support the need for measuring the absorption by each phase individually. Although no published work could be found which showed that the mechanisms of intracutaneous and systemic absorption were either similar or different, a theoretical treatment of the subject suggests that absorption by the two phases might be expected to differ in certain situations.

There appears to be general agreement that the routes by which drug molecules can be absorbed percutaneously are through the intact epidermis (transepidermal) and through the skin appendages (2,34,37,38). These potential avenues have been presented by Griesemer as shown in Figure 2 (1).

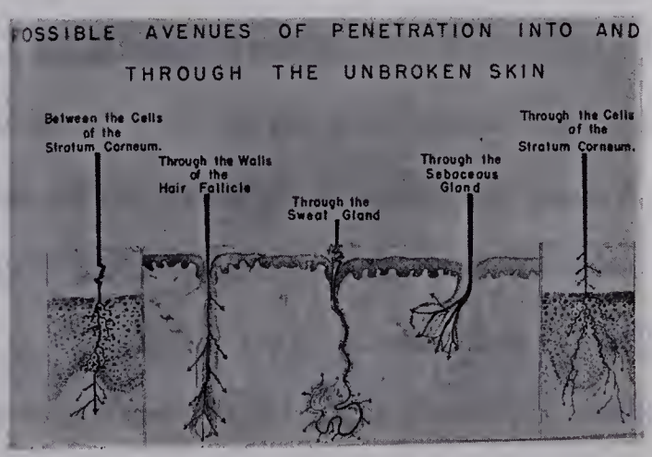


Figure 2. From Griesemer, R.D., Soc. Cosmetic Chemists, 11, 81 (1960).

Transepidermal absorption requires that the drug encounter the surface film, and then progress through the intact epidermis and part of the dermis respectively before it reaches the blood vessels. There are numerous supporters for this route being the major pathway for absorption (1,26,40,41). Other workers have demonstrated that absorption can also occur through the walls of hair follicles and sebaceous glands (42, 43,44). The role of sweat ducts and glands in transappendageal absorption is more controversial. Rein (45), Abramson and Gorin (46) and Abramson and Engle (47) supported the sweat ducts as an avenue for penetration but other investigators (42,48,49) have discounted their effectiveness in this respect. The consensus appears to be that the hair follicles and sebaceous glands (pilosebaceous apparatus) are the most probable path of entry. Drugs penetrating into the skin through the pilosebaceous apparatus must encounter in turn the sebum in the follicles and glands, the epidermal walls of the appendages and varying degrees of dermal tissue before reaching the blood vessels. Both the transepidermal and pilosebaceous avenues are usually available to topically applied preparations. It is likely that the relative importance of one over the other as the major route of entry for a specific drug preparation would be determined by the chemical and physical properties of the drug and the type of vehicle in which it was presented (16,37,38). The important feature of these two routes with regard to intracutaneous and systemic absorption is that drug absorption by both phases can be realized since living epidermal and dermal

tissues as well as blood vessels are common to both pathways.

Mathematical relationships predicting the effects of formulation and biological factors on percutaneous absorption have been presented by Higuchi (37). This theoretical treatment of the processes believed to be involved in absorption also included a consideration of the relative distribution of drug in various parts of the skin after absorption from different systems. For this reason the above reference was used in the present discussion as a basis for postulating situations where systemic absorption may not be proportional to intracutaneous absorption.

The location of the rate determining step to the absorption of a drug from a topically applied vehicle into and through the skin basically differentiated the relationships presented by Higuchi (37). In the first situation considered, the rate determining step to absorption occurred across the "major barrier" of the skin. Assuming that the drug passed through the absorption steps by passive diffusion and that the vehicle containing the drug did not appreciably affect the skin, Higuchi (37) developed the following approximate relationship for an idealized system between the steady state rate of penetration across the "major barrier" and the properties of a fairly water soluble drug:

$$\frac{dq}{dt} = (P.C.) \quad \frac{(\text{Conc. of Drug})DA}{L} \qquad \text{(Eq. 1)}$$

where: $\frac{dq}{dt}$ = the rate of penetration across the barrier;

P.C. = the effective distribution coefficient of the drug between the vehicle and the barrier of the skin;

Conc. of Drug = the concentration of drug in the vehicle;

D = the diffusion coefficient of the drug in the barrier phase;

A = the plane of area across which diffusion is occurring;

L = the thickness of the barrier phase.

He showed that this equation may also be expressed in terms of the thermodynamic activity of the drug in the vehicle:

$$\frac{dq}{dt} = \frac{a}{\gamma} \quad \frac{DA}{L} \qquad \text{(Eq. 2)}$$

where:

a = the thermodynamic activity of the drug in the vehicle;

γ = the effective activity coefficient of the drug in the skin barrier phase.

The activity gradients of drug resulting in the vehicle and the skin when the rate determining step to absorption occurs across the "major barrier" were schematically presented as in Figure 3. This relationship indicates that the vehicle can provide the drug faster than the carrier can accept it and that the lower tissues can accept virtually all of the drug which the barrier permits to pass. The lower tissues referred to in this case are the epidermal and dermal tissues

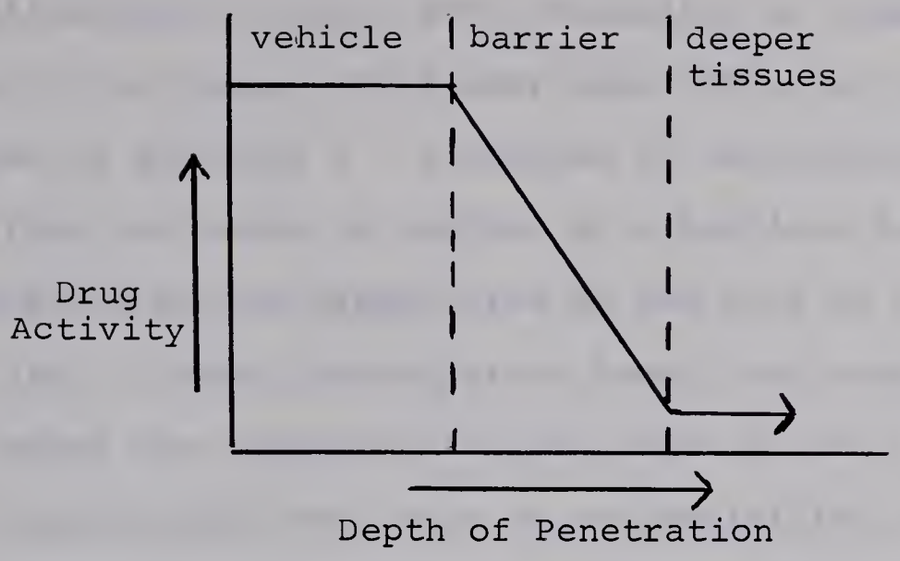


Figure 3: Schematic diagram showing changes in drug activity at different depths of penetration (as adapted from Higuchi (37)).

and the blood vessels. Therefore the relative distribution of penetrating drug among these tissues would determine whether intracutaneous and systemic absorption would be proportional and whether absorption by either phase would approximate absorption as predicted by equation 2. If the majority of drug penetrating through the barrier should be removed by the blood as suggested by Scheuplein (38) or, conversely, if the penetrating drug initially accumulates in the epidermal and dermal tissues before reaching the blood supply as was indicated by the work of Treherne (21), absorption by the systemic and intracutaneous phases would not be expected to be proportional. If one phase were to receive most of the penetrating drug in either

of these situations it would seem reasonable to assume that absorption by that phase would best agree with the absorption as predicted by equation 2. A problem in extrapolating absorption data from one phase to another in situations such as postulated above may be exemplified by the work of Carson and Goldhamer (50). These investigators found that upon emulsifying tritiated water the absorption of the water by skin tissues was increased considerably over that of non-emulsified water. The effect of emulsification, however, decreased the absorption of the tritiated water by the blood. Therefore, an interpretation of the effect of emulsification upon percutaneous absorption by blood measurements alone would have resulted in an erroneous conclusion of reduced absorption. Since the "major barrier" has been demonstrated to be the rate determining step to the percutaneous absorption of water (25,43), these results would appear to support the need for intracutaneous as well as systemic absorption measurements of all compounds whose absorption is similarly limited by this region.

Higuchi (37) also predicted that the rate determining step to the percutaneous absorption of a drug may develop in the applied vehicle when the drug is dispersed throughout the preparation in a finely suspended form. According to this theory the rate at which drug is released from the vehicle would be less than the rate at which the skin could absorb the drug and the critical absorption gradient would be in the vehicle as shown in Figure 4. On this basis, Higuchi (51) derived the following equation describing the variables affecting the

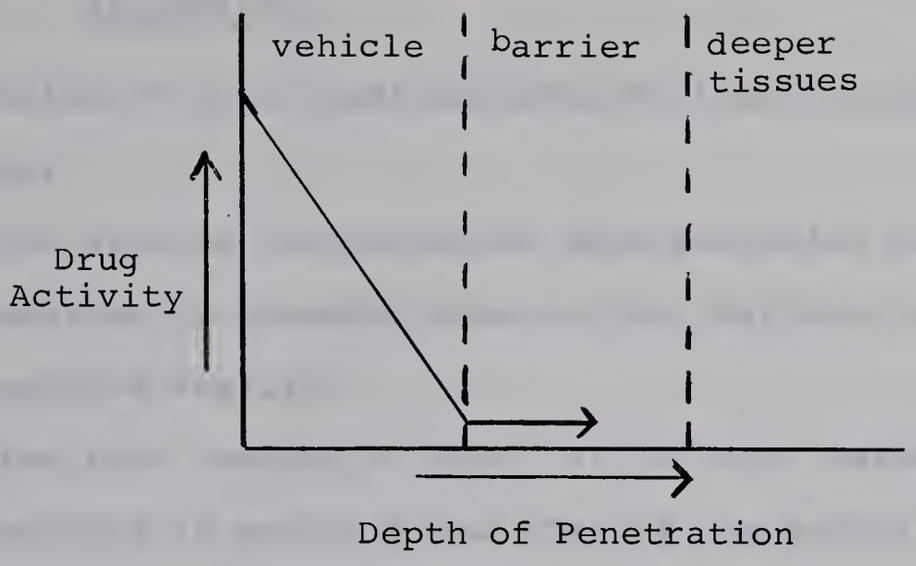


Figure 4: Schematic diagram showing activity gradient when the rate determining step is in the vehicle (as adapted from Higuchi (37)).

rate of release of solid drugs suspended in a vehicle:

$$\frac{dq}{dt} = 1/2 \sqrt{\frac{D(2A - C_s)C_s}{t}} \qquad \text{(Eq. 3)}$$

where:

$\frac{dq}{dt}$ = the rate of drug released per unit area of surface exposure;

A = the total concentration of drug in the vehicle (suspended and dissolved drug);

C_s = the solubility of the drug in the external phase of the vehicle;

D = the diffusion constant of the drug molecule in the external phase of the vehicle;

t = the time at which the amount of drug released is

determined.

The derivation of this equation involved the following basic assumptions:

(a) the size of the suspended drug particles are much smaller in diameter than is the thickness of the applied vehicle;

(b) the total amount of drug (A) per unit volume of vehicle is much greater than is the solubility of drug (C_s) per unit volume;

(c) the surface to which the preparation is applied functions as a perfect sink for the released drug.

For the case where there was much more solid drug present than required to saturate the external phase of the vehicle ($A \gg C_s$), it was shown that equation 3 could be simplified to

$$\frac{dq}{dt} = \sqrt{\frac{ADC_s}{2t}} \qquad \text{(Eq. 4)}$$

Since the skin probably does not function as a perfect sink, intracutaneous and systemic absorption measurements would not be expected to agree initially with the rate of release and consequently the rate of absorption predicted by equation 4. However, once the rate of drug penetration across the "major barrier" reaches a steady state (Figure 4), absorption by the lower tissues should approximate the value predicted. Whether absorption by the intracutaneous and systemic phases would be proportional and whether absorption by either phase would agree with the absorption predicted by equation 4 would depend again on the relative affinity of the penetrating drug for

each phase. There is evidence that absorption by the two phases of a drug suspended in systems such as described by equation 4 was not proportional. Intracutaneous absorption as determined by Plein and Plein (52) indicated that salicylic acid was absorbed to a greater extent from a petrolatum ointment than from an absorption base. Plasma measurements, however, showed that salicylic acid was probably absorbed systemically to a greater extent from the absorption base than from the petrolatum ointment.

The relationship of variables considered to influence percutaneous absorption such as developed by Higuchi (37) may be used by formulators as a guide for designing drug preparations which would enhance optimum absorption. The value of such a theoretical guide, however, should be verified by experimental studies. Furthermore, since there appears to be reasonable doubt as to whether the proposed variables would affect intracutaneous and systemic absorption to the same degree, these experimental studies should be undertaken for both phases. The accuracy of such an evaluation could be expected to depend on the degree of sensitivity of the methods available for measuring absorption.

C. Methods of Measuring Percutaneous Absorption

The objective of measuring percutaneous absorption should be to obtain a quantitative evaluation of the rate and extent of penetration by a topically applied drug into and through the skin. Since the degree of absorption in both the intracutaneous and systemic phases are of interest in dermat-

ological therapy, absorption by both phases should be measured. A variety of methods for measuring percutaneous absorption has been used with varying degrees of success. These methods are presented in the following discussion according to the absorption phase which they measure.

In vitro Methods

All procedures using inanimate media for measuring absorption may be classified as *in vitro* methods. The most popular approaches to such measurements have been to determine drug release from a vehicle either into a medium such as agar gel or through a medium such as cellulose film. Models developed by Wood, Rising and Hall (53) and by Billups and Sager (54) appear to be satisfactory for measuring the release of a drug by a vehicle and consequently the effect of formulation factors which might influence this release. They are inadequate, however, for evaluating the effect which such factors may have on the subsequent absorption of the released drug by the skin. The complex chemical, physical and biological properties of living skin preclude adequate simulation by *in vitro* models (55). Therefore, it is felt that intracutaneous and systemic absorption studies must be performed on an *in vivo* basis.

Systemic Measurements

A quantitative method for measuring systemic absorption should be capable of determining the rate at which a topically applied drug penetrates into the circulatory system of the body. Methods which measure penetration into this phase are commonly

referred to as methods for measuring percutaneous absorption. This reference is unfortunate since it may imply that systemic absorption measurements are also representative of intracutaneous absorption. A review of such methods indicated that they did not necessarily represent intracutaneous absorption. In some cases, they may even have significant limitations as quantitative methods for measuring systemic absorption.

The appearance of pharmacological effects which are characteristic of penetrating drugs is one method which has been used to interpret percutaneous systemic absorption. Vasodilation (56), vasoconstriction (57), degree of anesthesia (58,59), reduction in pain threshold (60,61), increased blood pressure (62), convulsions (63) and death (64) are among the responses which have been measured to determine the degree of drug penetration through the skin. There are limitations to this approach, however, as a quantitative measure of systemic absorption. The most obvious limitation is that the absorption of only those drugs which elicit a measurable physiological response can be studied in this manner (65). Furthermore, even if the drug is capable of effecting a significant response the magnitude of such a response is difficult to calibrate and quantitative results are usually replaced by a yes-or-no answer to the question of absorption (55). Another pitfall to this approach may be encountered if the drug should be absorbed but only in a concentration below the minimum threshold required to register a response (66). Finally, unless the amount of drug which is metabolized, stored and excreted is

known relative to the total amount which penetrates, a quantitative evaluation of the rate of systemic absorption by this method would not be reliable (65).

Perhaps the most popular procedures used to measure systemic absorption have been the analysis of blood (67,68,69, 70,71), urine (72,73,74,75,76) and target organs (77,78,79,80, 81) for presence of the penetrating molecule. If the drug should be selectively absorbed by any one of these receptors on its route through the body or if it should produce a toxic reaction specific to a receptor, analysis of the receptor for drug content becomes important. However, caution must be exercised in accepting the drug concentration in any one area as an estimate of total drug penetration through the skin. For example, the amount of drug in the blood at any given time would be the net result of the amount which had entered minus the amount which had been removed. To be representative of the quantity of drug which penetrated through the skin, therefore, the blood concentration must be corrected for the amount of drug which may be stored in various tissues, metabolized and excreted. Griesemer, Blank and Gould (82) have demonstrated an approach for making such a correction. They injected a known concentration of drug intravenously into an animal and then analyzed the blood for the drug after various time intervals. In this manner they calibrated the animal for percutaneous systemic absorption studied. They compared the amount of drug which left the blood due to storage, excretion and metabolism to the amount of drug which remained in the blood.

An alternative to correcting for such variables would be to study drug penetration through isolated skin. Treherne (21) employed a method in which excised skin was placed in a cell over a receptor solution, the drug preparation was applied to the surface of the skin and subsequent penetration was measured by analyzing the receptor solution for drug content. A modification of this procedure was demonstrated by Ainsworth (65) with the objective of more closely approximating the dynamic removal of the penetrant by the blood vessels. He facilitated the recovery of the penetrating substance by perfusing the intact blood vessels below the area of isolated skin with a physiological solution. The solution passing through the vessels was then sampled periodically for the test substance. A criticism of using isolated skin in perfusion studies could be that the barrier properties of isolated skin may not be the same as those of intact skin. Reports by Tregear (2), Choman (44) and Stoughton (83) suggest that this criticism is valid.

Intracutaneous Measurements

Methods for measuring intracutaneous absorption should be directed at determining the amount and distribution of drug in this phase at various time intervals rather than at the rate with which the drug penetrates into this phase for the following reason. The rate of entry may not necessarily represent the amount of drug in the living epidermis and dermis since some drug probably will pass from these tissues into the circulatory system. The concentration and distribution of

drug in this phase are important if it is assumed that the primary objective of initiating intracutaneous absorption is to achieve the therapeutic effect of the drug in this area. A major obstacle to quantitating drug absorption by this phase is in the separation of the drug from the tissues for analysis. This obstacle may be overcome largely by the use of radioisotope-labeled drugs which can be detected in the tissues without separation (44). Radioisotopes are also advantageous in that they offer a high sensitivity of measurement (84). This factor is particularly important in intracutaneous absorption investigations where the amount of drug absorbed as compared to the amount applied is usually very small (55).

Despite the advantages offered by radioisotopes, the methods which have been used to measure intracutaneous absorption with this technique still appear to have limitations in providing quantitative, reproducible results. Witten (85), Malkinson (86) and Ainsworth (65), among others, have studied the penetration of radioactive-labeled drugs into the skin by measuring the disappearance of radioactivity from the surface. These basic procedures involved applying the labeled drug to the skin, counting the radioactivity on the surface with a Geiger counter and then relating the decrease in surface counts at various times to the amount of drug being absorbed. Several shortcomings prevent this technique from qualifying as a quantitative, reproducible method for measuring intracutaneous absorption. Variables in counting the radioactivity on the surface may produce large experimental errors in this pro-

cedure (55). For example the geometry or position of the subject relative to the counter must be kept constant. A small movement by the subject away from the counter would result in a significant decrease in registered counts. Such a decrease could be erroneously interpreted as representing an increase in absorption. Since the amount of drug which penetrates into the skin is usually much less than that present on the surface, errors of this nature may prevent the accurate measurement of absorption (55). A loss of radioactivity from the surface could also result from the drug penetrating into the stratum corneum and into the blood system, neither of which represent intracutaneous absorption by the terms of reference previously stated (see p. 1).

Histological examination of skin sections for the presence of absorbed drug also has been a popular approach to measuring intracutaneous absorption. Strakosch (87) accepted keratolysis in such sections as evidence of the absorption of salicylic acid. Although this method may be effective for determining the presence and depth of penetrant in the skin it does not permit a quantitative evaluation of absorption (55). Examination of skin specimens for tracers indicating drug absorption also has been used. Dyes (88,89,90), fluorescent compounds (88,90) and radioactive isotopes (91,92,93) are among the tracers which have been used to detect drug molecules. As Blank (55) has pointed out, however, absorption data from studies using tracers should be accepted with caution since the tracer may become detached from the drug

molecule during the absorption process and consequently not be representative of the amount of drug present. Again, the use of stable radioactive-labeled drugs has largely overcome this problem of separation. The work of Choman (44) on autoradiography exemplified the use of this technique in investigating the absorption of drugs by the skin. Although autoradiography provides information relative to the depth and route of penetration it has not been demonstrated as yet to be more than a qualitative method for studying intracutaneous absorption.

The most logical method for quantitatively measuring intracutaneous absorption would appear to be analysis of skin tissues for drug content. Problems complicating this apparently simple approach have to date precluded the development of an adequate refinement of such a procedure. Analysis of the complete skin specimen exposed to the drug preparation introduced the question of whether unabsorbed drug was being measured as well as absorbed drug. Such an analysis would include a measure of any drug present on the surface and in the dead superficial tissues of the skin. Under these conditions the data obtained would not present an accurate measurement of actual absorption. As proposed by Plein and Plein (52) a solution to this problem would be to thoroughly remove the unabsorbed drug before analysis. Even if this could be done efficiently the vigorous treatment required could influence the amount and depth of normal drug absorption. In addition, analysis of whole skin cannot be expected to present

a picture of drug distribution throughout the tissues concerned (55). On this basis, this approach must be considered to be less than ideal.

Blank and Gould (94) demonstrated a procedure which they claimed overcame the difficulty of differentiating between absorbed and unabsorbed drug. Refining a method developed by Flesch (95) they separated the stratum corneum, the remaining epidermis and the dermis in hairless skin by stripping off each successive region with pressure-sensitive tape. This procedure was proposed for analysis of drug present in the epidermal and dermal tissues respectively. The physical processes of washing, heat and pressure required to effect the separation of the tissues for analysis could be sufficient to again produce an abnormal drug distribution picture. For these reasons the reliability of such an analytical technique could be questioned.

Perhaps the most useful attempt at quantitating drug absorption by the skin as well as determining the distribution of drug through the skin was introduced by Sheinaus, Christian and Sperandio (96). Using an apparatus to control the application of radioactive-labeled drug preparations to specific surface areas of the skin, they studied the effect of the enzyme hyaluronidase on intracutaneous absorption. Skin sections were recovered at successive depths from the treated specimen by slicing the skin parallel to the surface with a freezing microtome. The depth and extent of drug absorption were then determined by analyzing the serial sections of skin for radioactivity. A similar procedure was employed by

Carson and Goldhamer (50,97) in evaluating the relative absorption of tritium-labeled water from emulsified and non-emulsified preparations. Although the results presented by both research groups indicated trends differentiating treatments and controls, variation among replicates was very large. In fact, values in the order of several hundred per cent were obtained. Unfortunately no statistical analysis was presented by either group. This lack of reproducibility critically limited the value of these procedures as quantitative methods for measuring the effects which formulation factors may have upon drug absorption by the skin. Nevertheless, these procedures presented a practical approach to the development of a method for measuring intracutaneous absorption. If the experimental error could be reduced by refining the technical variables involved, a method enabling quantitative, reproducible data could be expected to result.

STATEMENT OF PROBLEM

The major objective of this investigation is to develop a method for quantitatively measuring the effects of formulation factors on the intracutaneous absorption of drugs. The importance of measuring such absorption by the skin as well as measuring systemic absorption through the skin will be determined experimentally by comparing absorption results from each phase as a function of rest time and of concentration. These effects of the rest time and concentration variables on absorption by each phase will be compared to predicted values derived theoretically.

EXPERIMENTAL METHODS AND DISCUSSION

Technical variables involved in the application of a drug preparation to the surface of the skin as well as in the subsequent recovery of skin samples for the analysis of drug absorption were expected to contribute to the experimental error of a method designed to quantitatively measure intracutaneous absorption. The following discussion describes the steps taken to identify and control these variables.

A. Drug Preparation Used

The choice of drug was influenced by availability in a stable, radioisotope-labeled form and by the ability of the molecule to penetrate into and through the skin. The label preferred was either carbon-14 (C^{14}) or tritium (H^3) since these labels are most common to organic compounds. Therefore any procedures developed with them would be applicable to later absorption studies involving the majority of drugs used in dermatological practice. Salicylic acid (carboxyl-C^{14}) met these specifications as there was evidence that salicylic acid

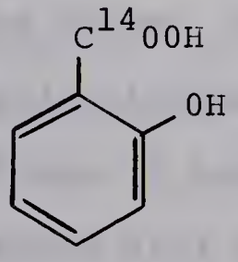


Figure 5: Salicylic acid showing position of C^{14} label.

penetrated into and through the skin (52,70,87,98,99). Furthermore, since salicylic acid is used in modern dermatology as a keratoplastic and a keratolytic agent, it was selected as the drug of choice for this investigation. The labeled salicylic acid was obtained in crystal form from Nuclear-Chicago* in a specific activity of approximately 15.5 mc/mM. It was later dissolved in ethanol and part of this stock solution was diluted with non-radioactive salicylic acid to produce a specific activity of approximately 18 μc/100 mg of drug in one ml of solution (solution I). The remainder of the stock solution was diluted in a similar manner to produce a specific activity of approximately 90 μc/100 mg of drug in one ml of solution (solution II). Both solutions were placed in 10 ml vials sealed with rubber stoppers under aluminum caps. The vials were then stored in light-proof containers until the solutions were required for incorporation into the dermatological vehicles.

Shelmire (98) stated that the primary requirement for topical therapy is that the drug presented in a vehicle must be released to the surface of the skin at an adequate rate. He also pointed out that although the vehicle itself may not penetrate the skin to any extent, there may be a marked difference in the clinical effectiveness of a drug depending upon the vehicle employed. Since it appeared that the properties of the vehicle may influence drug absorption, such properties would have to be kept constant if absorption from a given preparation was to be reproducible from experiment to experiment. The distribution of drug throughout the vehicle and the con-

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* Nuclear-Chicago Corporation, Des Plaines, Illinois, U.S.A.

sistency of the resulting preparation were considered as important variables which could influence the amount of drug presented and also the degree of drug contact with the skin.

In searching for a satisfactory vehicle, preliminary testing indicated that salicylic acid was only sparingly soluble in commonly used dermatological vehicles. The high viscosities of such systems made quantitative separation of suspended matter a difficult procedure. For that reason the relative solubility of salicylic acid in these vehicles was estimated by microscopic examination for undissolved crystals.

Since equation 4 (see p. 20) suggested that absorption of drug should increase with an increase in the dissolved portion of the drug, efforts were continued in this direction until a four to one ratio of Modulan* to olive oil was selected as satisfactory (preparation A). Although an increase in the olive oil fraction appeared to increase the solubility of the salicylic acid proportionally, an increase in fluidity also occurred, eventually producing a preparation which was too fluid to confine to a specific surface area on the skin. For that reason the four to one ratio was selected as the best compromise between solubility and consistency. A 10 per cent w/w concentration and approximately 18 μc/Gm specific activity of salicylic acid was prepared in this vehicle by the following

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* Modulan is a registered trademark of the American Cholesterol Products, Inc., Edison, New Jersey, U.S.A. It is described as an acetylated lanolin product designed to more closely approximate human skin lipids than does lanolin itself.

procedure. One ml of solution I (see p. 33) was added to each 900 mg of vehicle used. The ethanol was removed by evaporation and the salicylic acid then was incorporated into the vehicle by thorough levigation on an ointment slab. The finished preparation, which was primarily a suspension, was sealed in 2.5 ml disposable syringes and stored in the refrigerator. Uniformity of drug distribution throughout the vehicle was confirmed at the time of manufacturing and at each time the preparation was used.

Hydrophilic Ointment U.S.P.* was employed as the second vehicle for salicylic acid (preparation B). In contrast to the oleaginous nature of the vehicle used in preparation A, this base was a more complex oil-in-water system. The problems associated with determining the solubility of salicylic acid in preparation B were similar to those encountered for preparation A. Microscopic examination again indicated, however, that both a 5 per cent and a 10 per cent w/w concentration of salicylic acid in preparation B were primarily in suspension form. Through use and subjective evaluation, the consistencies of preparations A and B appeared to be similar although no definite viscosity measurements were undertaken. The problem of keeping the consistency of preparation B constant during manufacturing and storage, however, was greater than that for preparation A because of the presence of an aqueous phase in the hydrophilic ointment.

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* The United States Pharmacopoeia, Seventeenth Revision, 1965, Mack Publishing Company, Easton, Pa., U.S.A.

With this problem in mind, the following procedure was adopted for making preparation B.

The ethanol solution of salicylic acid was added to the vehicle in a container selected to afford a minimal exposure of surface area to the atmosphere. The ethanol was then evaporated as rapidly as possible by passing a stream of air over the open container. When approximately 80 per cent (by weight) of the ethanol had been removed, the remaining preparation was placed between two sheets of Saran Wrap*. The incorporation of the drug into the vehicle was facilitated by manipulating the preparation between the sheets of Saran. The evaporation of the aqueous phase of the vehicle causing a change in the consistency of the preparation was reduced in this manner. The storage condition and uniformity testing schedule for preparation B were similar to those employed for preparation A (see p. 35). Two preparations representing different concentrations and different specific activities of salicylic acid in Hydrophilic Ointment U.S.P. were made. A 10 per cent (approximately 90 uc/Gm) product was prepared by adding one ml of solution II (see p. 33) to each 900 mg of vehicle used. A five per cent (approximately 45 uc/Gm) preparation was also made by diluting part of the 10 per cent preparation with equal parts of vehicle.

B. Experimental Animals Used

Preliminary studies to identify and quantitatively evaluate formulation factors which may influence percutaneous

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* Saran Wrap is a registered trademark of Dow Chemical Company, Midland, Michigan, U.S.A.

absorption can be carried out more readily and with better control on animals than on humans. Difficulties encountered in finding an adequate number of subjects to study; in establishing proper controls with subjects who participate on an out-patient basis and in drawing conclusions from subjective observations such as "improvement" or "no improvement" largely can be overcome with this approach. Since there is probably no animal with skin identical to that of man, experience gained from animal studies must subsequently be verified by detailed studies on humans. Nevertheless, such preliminary experience should prove to be valuable in the screening of formulation factors with potentially useful clinical application (100).

For this study the guinea pig was selected as the animal of choice because it was of a size suitable for the techniques involved and also because of its use in previous investigations on skin absorption (44,50,96,97,101). All experiments were performed on white, male, mongrel guinea pigs weighing between 600 and 800 Gm. The weight of each animal was determined and recorded immediately prior to use. The animals were maintained on a commercially available laboratory animal pellet diet which was in turn supplemented with greens. Skin from two anatomical sites representing hairy and hairless skin was included in the investigation. The dorsal surface of the animal was chosen as the site to represent hairy skin because of the ease of application to this area. Two regions were studied at this site in subsequent experiments, one on each side of the spine. The "hairless" or "bald spots" of the guinea

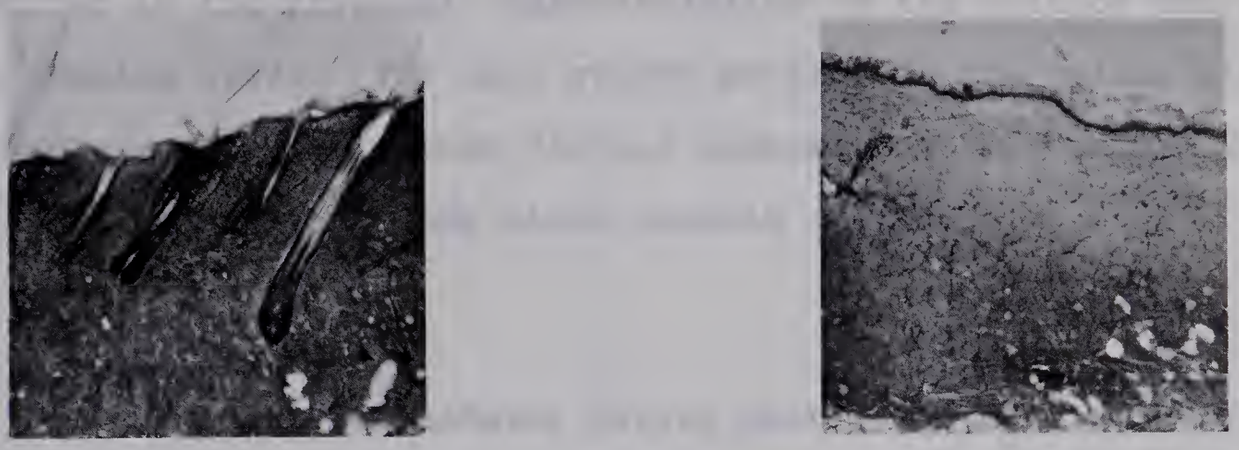
pig are located behind each ear (Figure 6) and have been described in detail by Zackheim and Langs (102). They reported these areas to be about 1.0 to 1.5 cm in diameter but tending to be larger in albinos than in colored animals. On the occasional animal some fine hair was found to be present

Figure 6. Bald area behind the ear of a white guinea pig as demarcated by black circle.

on these regions.

They observed that the major difference in the anatomical structure of the hairy and the bald skin was the absence of pilosebaceous apparatus in the latter (Figure 7).

Both "bald spots" on each animal were used for experiments on



A B

Figure 7. Photomicrographs of skin sections from hairy skin (A) and from bald skin (B) stained with hematoxylin and eosin (X40).

hairless skin. Any "bald spot" found to have fine hairs on it was rejected. The thickness of the skin layers in the specimens shown in Figure 7 was measured in each case with the aid of a calibrated microscope. The combined thickness of the stratum corneum and the remainder of the epidermis was found to vary from approximately 80 to 100 microns. The dermis was approximately 0.6 to 1.0 mm. Major networks of blood vessels were observed in skin samples from both sites in the subcutaneous region during the slicing of the samples parallel to the surface. Unfortunately, little information was found in

the literature dealing with the comparative anatomy of guinea pig and human skin. The absence of sweat glands in the skin of the guinea pig was perhaps the most noteworthy difference reported in the physical characteristics of the skin of the two species (103). The hair growth pattern in the guinea pig, however, was found to more closely approximate that in man than did hair patterns in some other animals (104).

C. Sedation Procedure

Animals were sedated during each experiment to facilitate the preparation of the skin and the application of medicament to the surface of the skin. Such sedation was also found necessary to prevent removal of the material by the animal and to reduce any stress produced in the animal due to handling. An inhalation technique was used similar to the one introduced by Hagen and Hagen (105). The animal first was placed in a glass dessicator jar containing anesthetic ether. As soon as the animal became unconscious it was transferred to the apparatus illustrated in Figure 8. Anesthesia was maintained by passing a stream of air (A,B) over the surface of liquid methoxyflurane (C) which, upon volatilizing, passed through the glass tubing (D,E) and then into the plastic funnel adapted to fit over the head of the animal. A second outlet (F,G) extended to the application apparatus so that anesthesia also could be maintained during application. The depth of anesthesia was increased by increasing the flow of air over the methoxyflurane and was reduced by decreasing this flow of air or by removing the

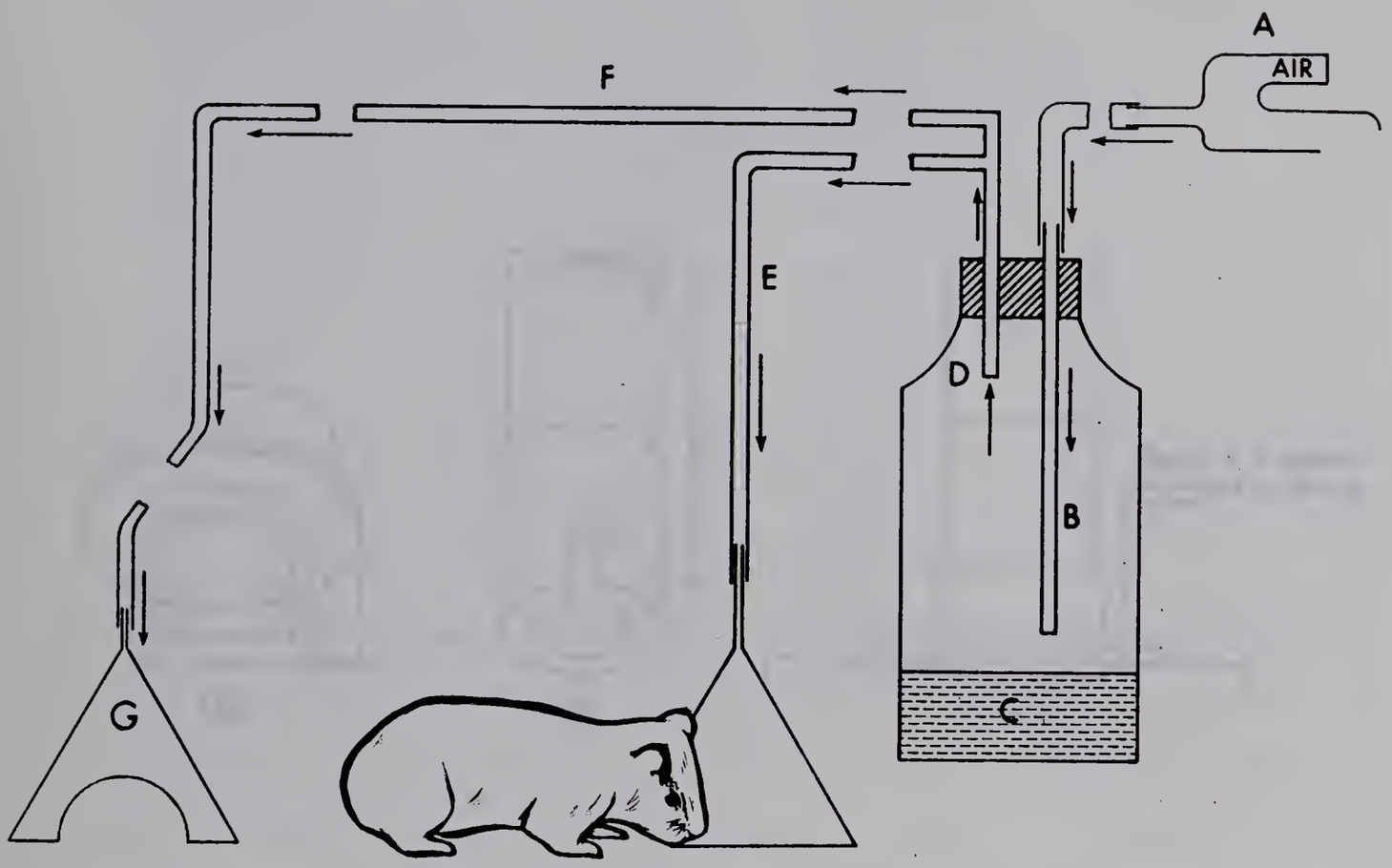


Figure 8: Apparatus for achieving sedation by inhalation.

funnel from the head of the animal. Satisfactory anesthesia was maintained by this procedure for varying lengths of time, with five hours being the maximum duration required for any experiment in this study.

D. Preparation of Skin for Application

The hair on the dorsal surface of anesthetized

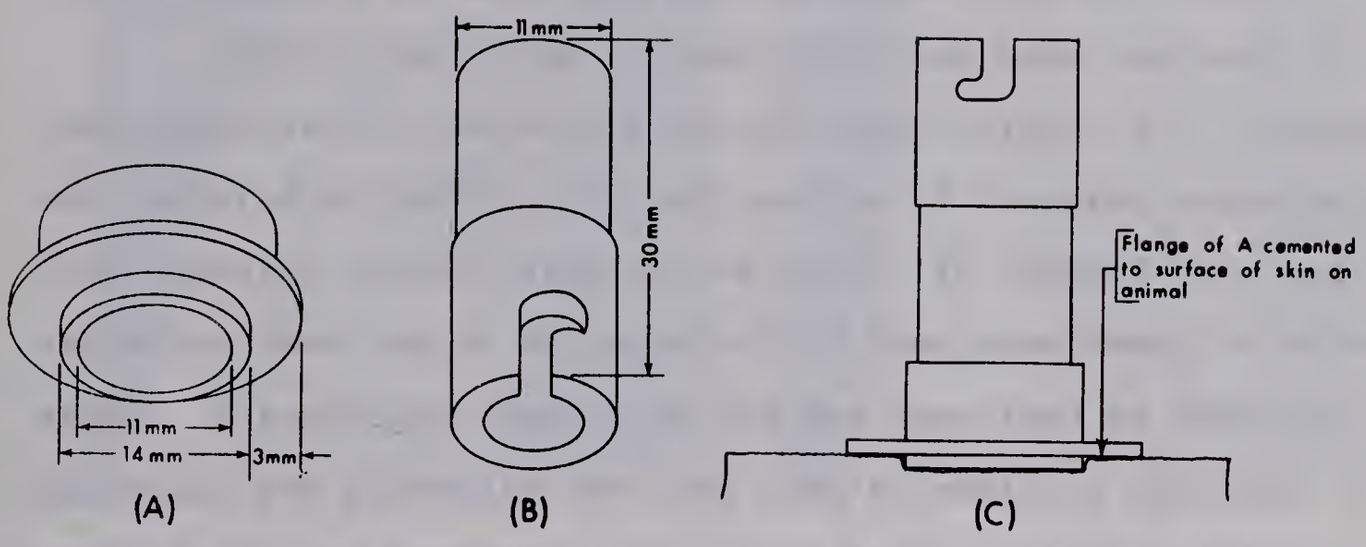


Figure 9: Detailed drawing of applicator assembly:
(A) - cylinder; (B) - applicator;
(C) - assembly in position.

animals was removed with an Oster* small animal electric clipper. When the bald spots were selected as the application sites, the hair surrounding these spots was removed in a similar manner. Coarse clipping was done with a number 10 blade followed by finer clipping with a number 40 blade. Initial irritation observed on the hairy sites due to clipping was not present on the bald sites since only the hair around

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* Oster is the registered trademark of the John Oster Manufacturing Co., Milwaukee, Wisconsin, U.S.A.

these areas had to be removed and clipper contact with the bald spot itself was avoided. One hour after clipping was completed the areas prepared for application were examined carefully for abrasions, scars and for hyperemia due to clipper irritation. Any application sites exhibiting such abnormal characteristics at this time were excluded from the study.

Since the degree of absorption has been proposed to vary according to the area receiving application (37), a device was required to confine the application of the drug preparation to a specific surface area on the skin. To control this variable the area had to be reproducible from experiment to experiment. A technique similar to the one described by Sheinaus, Christian and Sperandio (96) was used to obtain a constant surface area of exposure. Stainless steel cylinders (Figure 9(A)) were attached to the skin with household cement* over the intended sites of application (Figure 10). A flange, located one mm from the base of the cylinder and extending three mm beyond the external diameter of the cylinder, was designed to effect contact with the surface of the skin. Twenty mm circles were carefully marked around the intended sites with the aid of the open end of a test tube and an inked stamp pad. Cement was applied to the skin around the circumference of the circle so marked and also to the bottom of the flange on the cylinder. Approximately one minute after cementing, the

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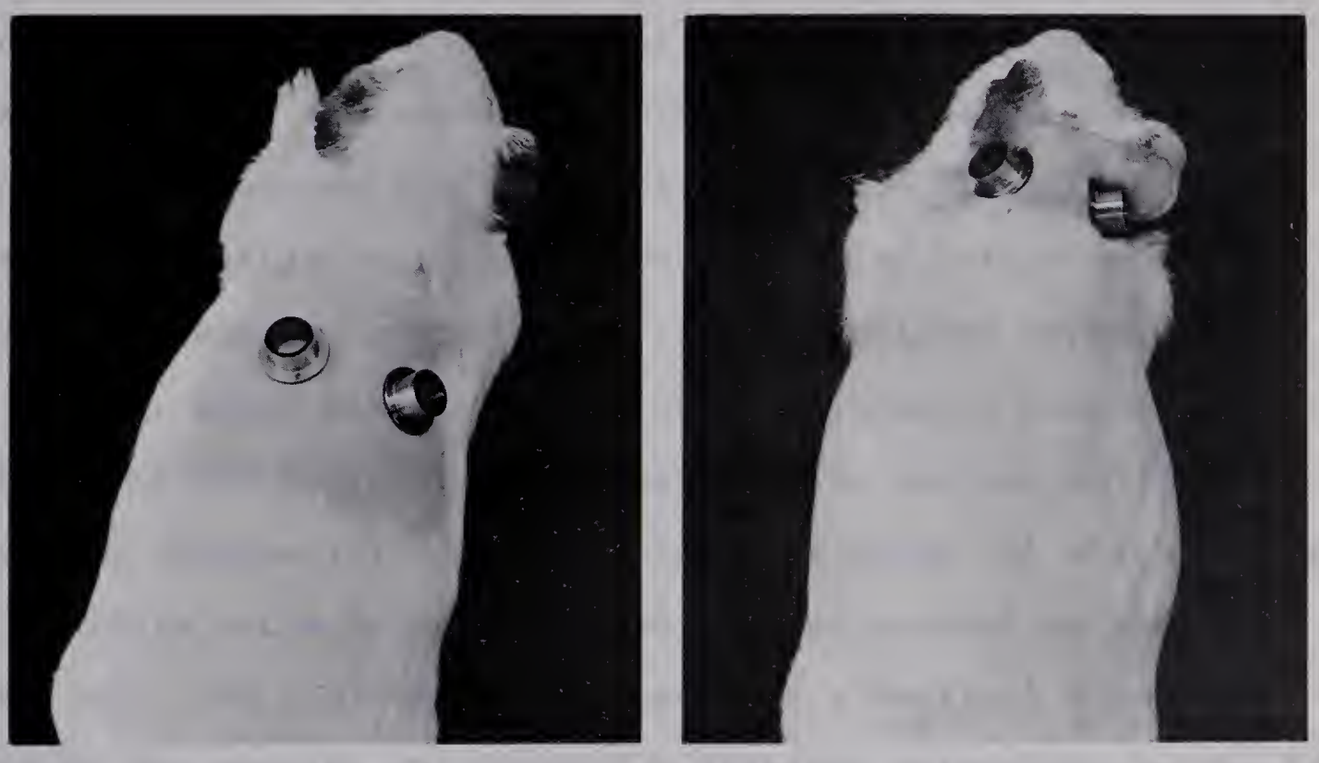
* Lepage's Pres-tite® Contact Cement, a product of Lepage's Ltd., Canada.

cylinders were found to be firmly attached to the two respective circles on the skin. This arrangement provided a specific



A B

Figure 10. Position of cylinders on hairy skin (A) and on bald skin (B).

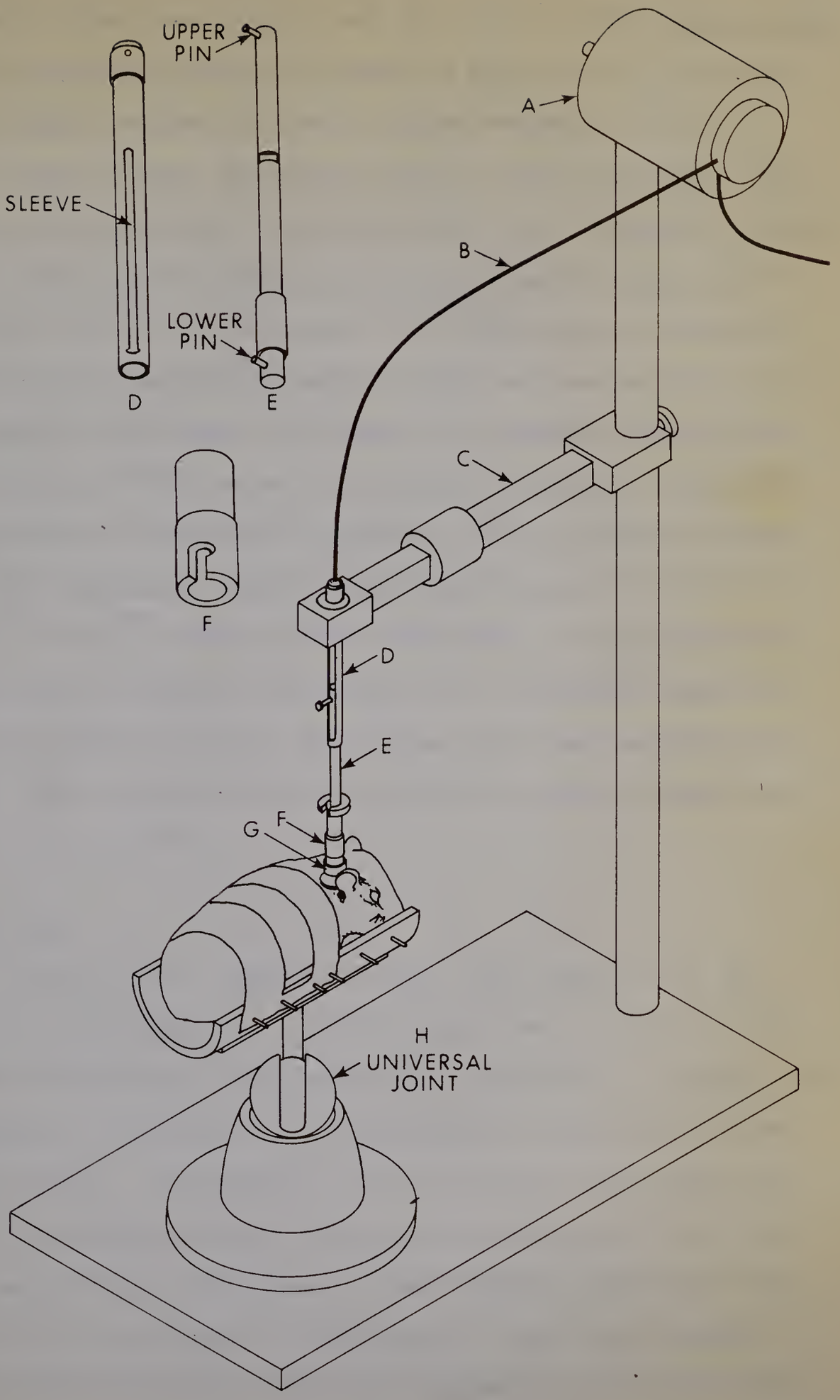
surface area (11 mm diameter) for application in the center of each cylinder. Teflon applicators (Figure 9(B)) were designed to introduce the drug preparation through the center of these cylinders and onto the surface of the skin (Figure 9(C)). The closely machined fit of the applicators in the cylinders permitted free rotation on the surface of the skin while at the same time resisting the loss of drug preparation between the applicator and cylinder.

E. Application of Drug Preparation

The viscous nature of the majority of vehicles used in

dermatological preparations requires that some degree of rubbing be used during the application of such preparations in order to obtain a satisfactory contact between the applied drug and the surface of the skin. Assuming that the degree of this contact influences the degree of absorption, rubbing became another variable which had to be controlled before consistent results could be expected from the contemplated experiments. A device, again similar to the one presented by Sheinaus, Christian and Sperandio (96), was used to produce uniform rubbing (Figure 11). It consisted of a motor (A) which rotated a flexible cable drive (B) which in turn rotated an aluminum rod (E). The rod could move freely in a vertical direction inside the housing (D) through a distance determined by the length of the sleeve cut through the housing wall. The upper pin on the rod prevented it from disengaging at this point. The drug preparation (20.0 $\pm$ 0.5 mg) was ejected from the storage syringe onto the surface of the applicator (F). This quantity of preparation was used since preliminary studies showed that, upon rubbing, 20 mg was slightly in excess of the amount required to completely cover the exposed 11 mm diameter skin surface. After loading with drug in this manner the applicator was attached to the lower end of the rod by means of a pin provided for this purpose. This assembly was then positioned directly over the cylinder cemented to the animal (G) and lowered into the cylinder until the preparation on the bottom of the applicator contacted the surface of the skin and the full weight of the rod and applicator rested on this surface. Such a

Figure 11: Drawing of complete application apparatus with animal in position.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stationary contact was maintained for five seconds before motor induced rotational rubbing was carried out at 30 r.p.m. for pre-determined lengths of time. Slight movements by the animal in the vertical direction such as those resulting from breathing were absorbed by the "floating" rod assembly. Other movements were restricted by a surgical elastic strap which secured the animal to the cradle. In this manner a constant rubbing pressure was maintained. A ball and socket joint (H) on the base of the cradle permitted the accurate positioning of the cradle and the animal to achieve the proper angle for the applicator to enter the cylinder. After rubbing was completed, the applicator was detached from the rod and left in position in the cylinder for the remainder of the experiment. This was done to enhance the contact of a constant amount of preparation with the skin. The animal was then removed from the cradle and returned to the anesthetic assembly described previously (see p. 40).

F. Rest Time

Since Stolar, Rossi and Barr (70) showed that the percutaneous absorption of salicylic acid was a function of the time in which the drug preparation remained in contact with the surface of the skin, it was considered important to control this factor. Accordingly, rest time in this study was taken as the period between the time of application and the time of sacrificing the animal. The sedated animal was sacrificed with an overdose of chloroform. The time required for procedures to prepare the skin samples for analysis after

sacrificing was not considered as being part of the rest period. It was recognized that some additional degree of absorption may have taken place during this period. However, since for all practical purposes the time required to prepare the skin samples for analysis was the same in all experiments, the increase in counts, if any, was considered to be constant.

G. Preparation of Skin Samples for Analysis

Skin sections, parallel to the surface of the skin and located at successive depths below the surface, were recovered and analyzed for radioactive salicylic acid. This method was selected to permit a quantitative comparison of the relative total absorption by skin samples from different experiments. In this way it was possible also to obtain an indication of the drug distribution gradient within each sample. To accurately compare relative drug absorption among samples, however, skin sections had to be of equal surface area and thickness. Furthermore, these sections had to be recovered from corresponding depths in the samples to be compared since it was expected that the level of radioactivity would increase with increasing proximity to the epidermal surface.

The following procedures indicate the difficulties encountered in recovering such sections.

Hairy Skin

The first approach (method I) at recovering uniform sections from various depths of the treated skin was patterned after that described by Sheinaus, Christian and Sperandio (96).

After sacrificing the animal the applicators were removed from the cylinders and the excess drug preparation was removed from the skin surfaces with cotton-tipped swabs. The dorsal skin of the animal was removed by cutting at least three cm outside of the treated sites. This skin was placed on a piece of waxed paper resting on a lead block. Circular skin punches were obtained using a stainless steel punch (19 mm diameter) on the two previously delineated circles on the skin representing the treated areas. Each skin punch was then transferred, epidermis up, to the stage of a Spencer freezing microtome* on which a drop of distilled water had been placed. Attachment of the punch to the block was made by rapidly freezing both with carbon dioxide. To obtain a level epidermal surface for slicing, a weight (approximately 20 Gm) was placed on the punch before freezing. The blade of the microtome knife** was positioned parallel to the epidermal surface of the punch and then lowered in 50 micron increments until it just contacted this surface. The punch was then sliced into 50 micron sections. These sections, with the exception of the first one, were individually transferred upon slicing to separate, serially numbered liquid scintillation counting vials*** which contained one

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* AO Spencer 888 Automatic Clinical Microtome and 930 Freezing Attachment, American Optical Co., Buffalo, N.Y., U.S.A.

** Razor blades were used for slicing skin sections in all experiments except autoradiography slicing where a 20 mm Spencer microtome blade was used. Blades were wiped after each section was sliced and were changed for each sample.

*** Standard 20 ml volume vials, #3323, Nuclear-Chicago Corp.

ml of digesting solution. The first section recovered was discarded since it was composed mainly of hair and residual drug preparation from the surface. Difficulties in leveling the surface of the skin punch and in adjusting the knife blade to be parallel to this surface often resulted in the second section also containing a portion of the surface residue. Therefore, although the second section was analyzed, it was not considered as being representative of drug absorbed. All skin sections were transferred from the microtome to the vials with a surgical dissecting needle. The vials containing the tissue and digesting solution were then capped and the contents were digested at 70° C for two hours. After this stage was completed, 10 ml of fluor solution was added and the samples were ready for counting. This method was used for all skin samples in experiments on hairy skin.

Bald Skin

The applicators and the excess drug preparation were removed from the bald spots in the same manner as before. The cylinders were left intact with the skin, however, and the treated sites were removed from the animal by cutting the skin immediately around the cylinders. This resulted in the cylinders being attached to a circular skin sample approximately 20 mm in diameter. The cylinder and skin combination was frozen, subcutaneous side down, on the quick freeze block of a microtome cryostat* for 30 minutes. This frozen assembly

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* International Model CTI microtome cryostat, International Equipment Co., Needham Heights, Mass., U.S.A.

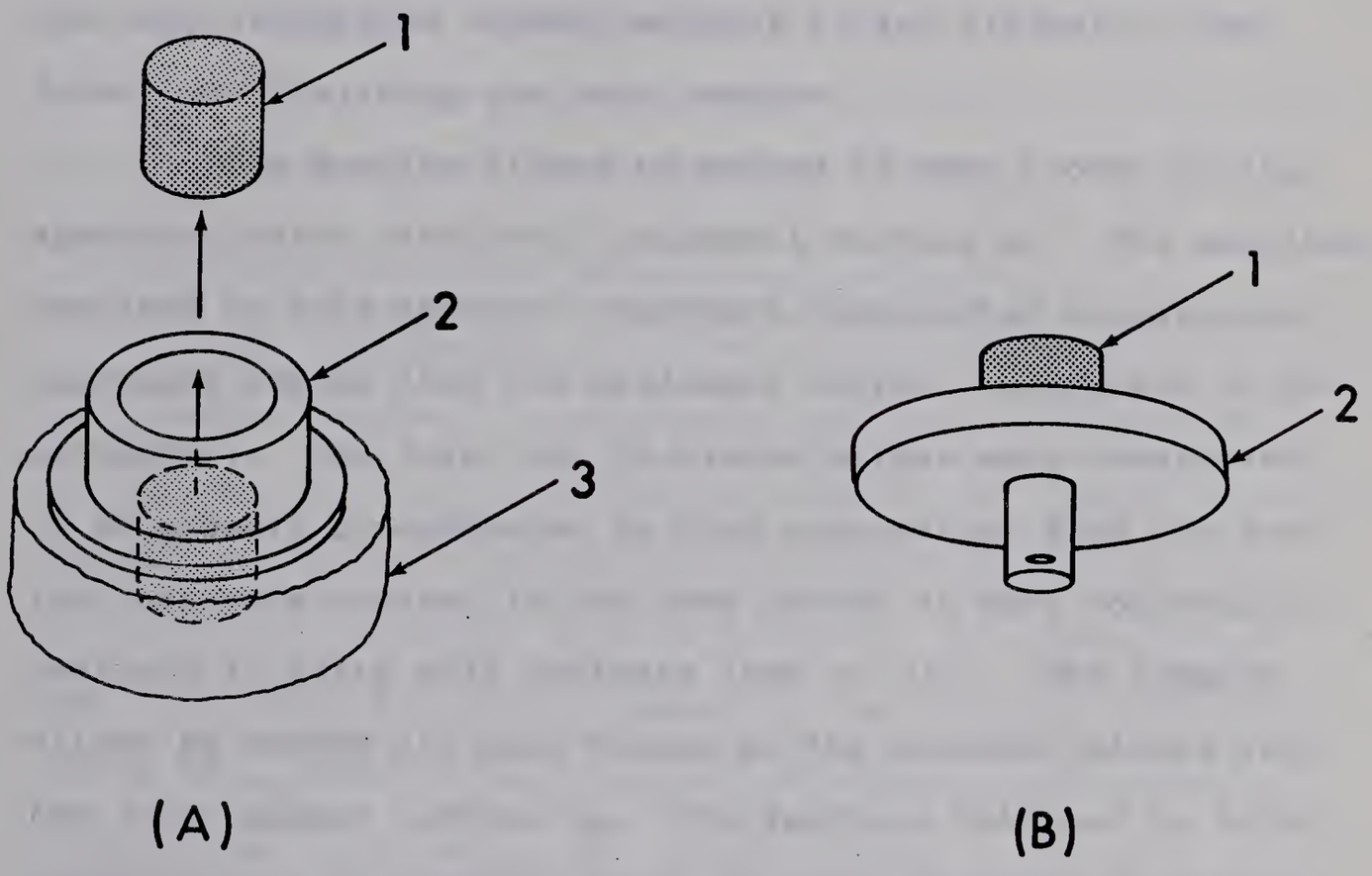

Figure 12: Details of: (A) Skin punch (1) recovered through cylinder (2) from skin (3); (B) Skin punch (1) on holder plate (2).

was then inverted, subcutaneous side up, on a lead block which was placed inside the cryostat. A punch of skin was removed through the center of the cylinder with an eight mm cutaneous punch (Figure 12(A)). The resulting punch was then placed on a drop of distilled water on a specimen holder plate and rapidly frozen to the plate (Figure 12(B)) on the quick freeze block. The plate and sample were then carefully adjusted and secured on the microtome in such a position that the cutting


1
2
3
4
(B)
(A)

blade could slice parallel to the surface of the sample. This procedure was used for methods II and III. It largely overcame problems experienced in method I in obtaining skin samples with inconsistent diameters and non-level epidermal surfaces. The only difference between methods II and III was in the direction of slicing the skin samples.

The samples sliced by method II were frozen on the specimen plates with their epidermal surface up. The sections obtained by this approach therefore represented successively increased depths from the epidermal surface (epidermis to subcutaneous). The first two 50 micron slices were considered to be grossly contaminated by drug preparation from the surface and were treated in the same manner as were comparable sections in hairy skin analysis (see p. 50). The samples sliced by method III were frozen on the specimen plates with the subcutaneous surface up. The sections obtained by this approach were in opposite order to those obtained by method II and represented decreasing depths from the epidermal surface (subcutaneous to epidermis). To determine the proximity of each section to the epidermal surface by this method, each punch was completely sliced until the ice below the surface was reached. The corresponding depth of each section was calculated in this manner by relating the section number to the subsequent number of 50 micron slices required to reach the ice. The two sections recovered immediately before reaching the surface were again considered to be contaminated by the drug preparation on the epidermal surface. Skin punches which

became detached from the specimen holder plate before sections 100 microns from the epidermal surface were obtained were not included in the study. Two sections of skin tissue (recovered by methods II and III) were placed in each counting vial. The digesting solution, the method of digesting and the fluor solution used for these samples were identical to those used for hairy skin samples obtained by method I.

H. Preparation of Blood Samples for Analysis

The purpose of measuring systemic absorption was to determine whether intracutaneous absorption as measured by the method developed in this investigation was proportional to systemic absorption as measured by a commonly used method. Cotty, Skerpac, Ederma, Zurzola and Kuna (71) demonstrated that the analysis of blood plasma was satisfactory for evaluating the systemic absorption of salicylates. Accordingly, blood plasma was used in the present study.

Approximately five ml of blood was removed from the animal by cardiac puncture immediately after the rest period. The blood sample was stored in the refrigerator until processing. Brodie, Udenfriend and Coburn (106) have shown that salicylic acid is stable in blood plasma when stored under such conditions. Later, the sample was centrifuged at 2,000 r.p.m. for 30 minutes. Duplicate 0.5 ml volumes of plasma were then pipetted into separate scintillation counting vials. After addition of a digesting agent to each, the samples were left to digest at room temperature for 12 hours. Fifteen ml of fluor solution was then added in preparation for counting.

I. Uniformity of Drug Preparations

Duplicate 50 mg samples were obtained from different parts of the drug preparation for each determination of the uniformity of drug distribution throughout the vehicle. The samples were weighed in counting vials and then 10 ml of fluor solution was added before counting. No additional digest was required to effect dissolution of the drug preparations. The average specific activities (DPM*/5 mg of drug preparation) together with the standard deviations of the various preparations were as follows:

10 per cent salicylic acid in preparation A (22 replicates) = 201,996 ± 4,778;

10 per cent salicylic acid in preparation B (24 replicates) = 1,009,541 ± 15,857;

5 per cent salicylic acid in preparation B (6 replicates) = 513,113 ± 13,354.

On the basis that the DPM of the 10 per cent salicylic acid preparation B represented 100.0 mg of drug per Gm of preparation, the relative DPM of the five per cent preparation therefore represented a concentration of 50.8 mg of drug per Gm of preparation.

J. Reagents

A 2N solution of potassium hydroxide in methanol was used to digest the skin sections. The solution was stored at

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* Disintegrations per minute

room temperature protected from the light. The normality was checked approximately every two weeks by the U.S.P. XVII method for standardizing 0.5N alcoholic potassium hydroxide. The plasma samples were bleached with five drops of 30 per cent hydrogen peroxide and then digested with four ml of NCS*, a quaternary ammonium hydroxide reagent.

The fluor solution used for the skin and drug preparation samples was similar to the one described by Petroff, Patt and Nair (107). It was prepared approximately once a month and was stored in the refrigerator. A primary and a secondary scintillation fluor were employed in the solution. These were 2,5-diphenyloxazole (PPO) and p-bis-{2-(5-phenyloxazolyl)}-benzene (POPOP) respectively. A secondary solvent, ethylene glycol monobutyl ether (butyl cellosolve), was added to the primary solvent of toluene to increase the miscibility of the skin tissue digest with the fluor solution.

FORMULA OF FLUOR SOLUTION

Toluene	-	850 ml
Butyl Cellosolve	-	150 ml
PPO	-	4.00 Gm
POPOP	-	0.05 Gm

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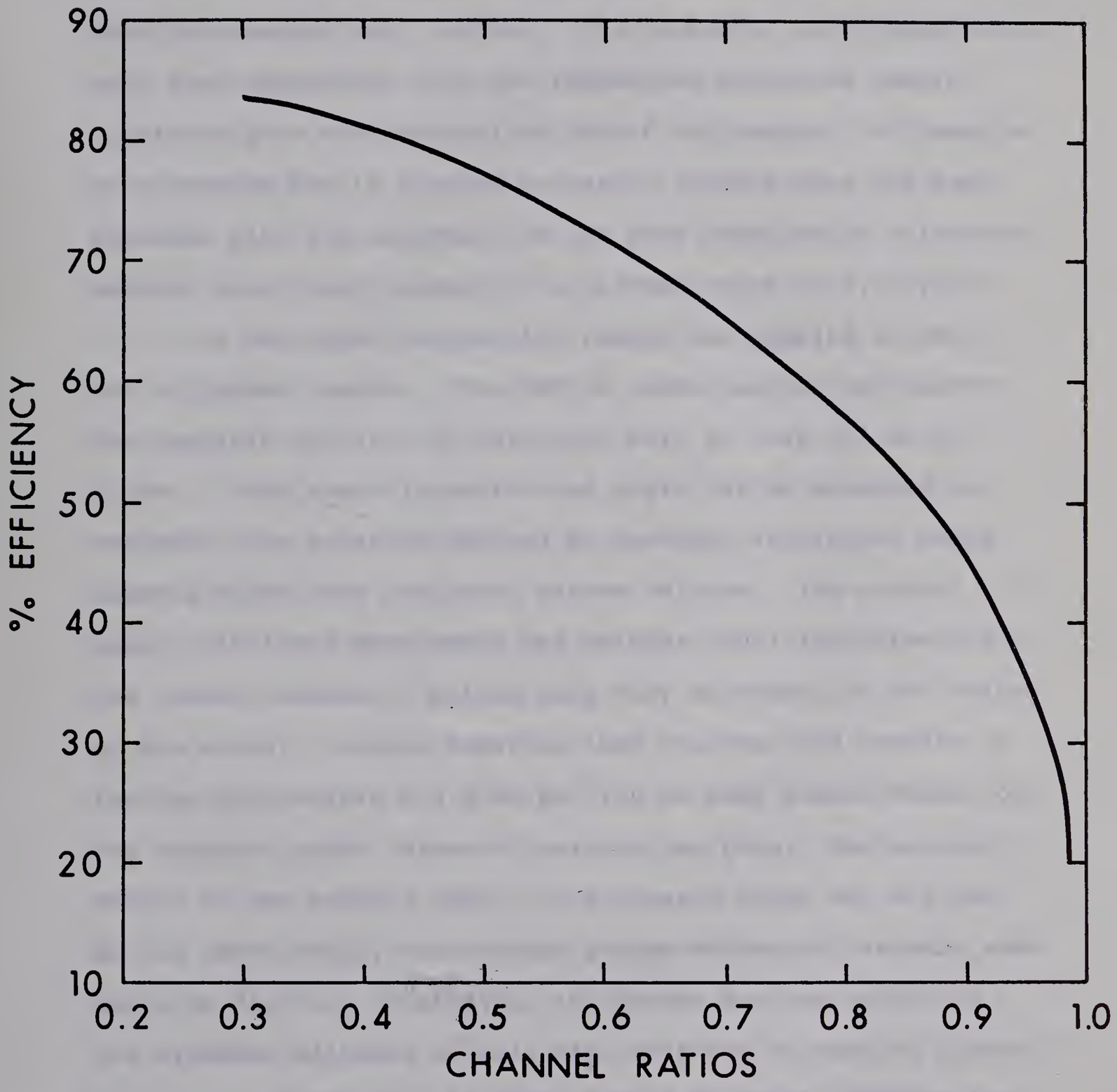
* NCS is a registered trademark of the Nuclear-Chicago Corp., Des Plaines, Illinois, U.S.A.

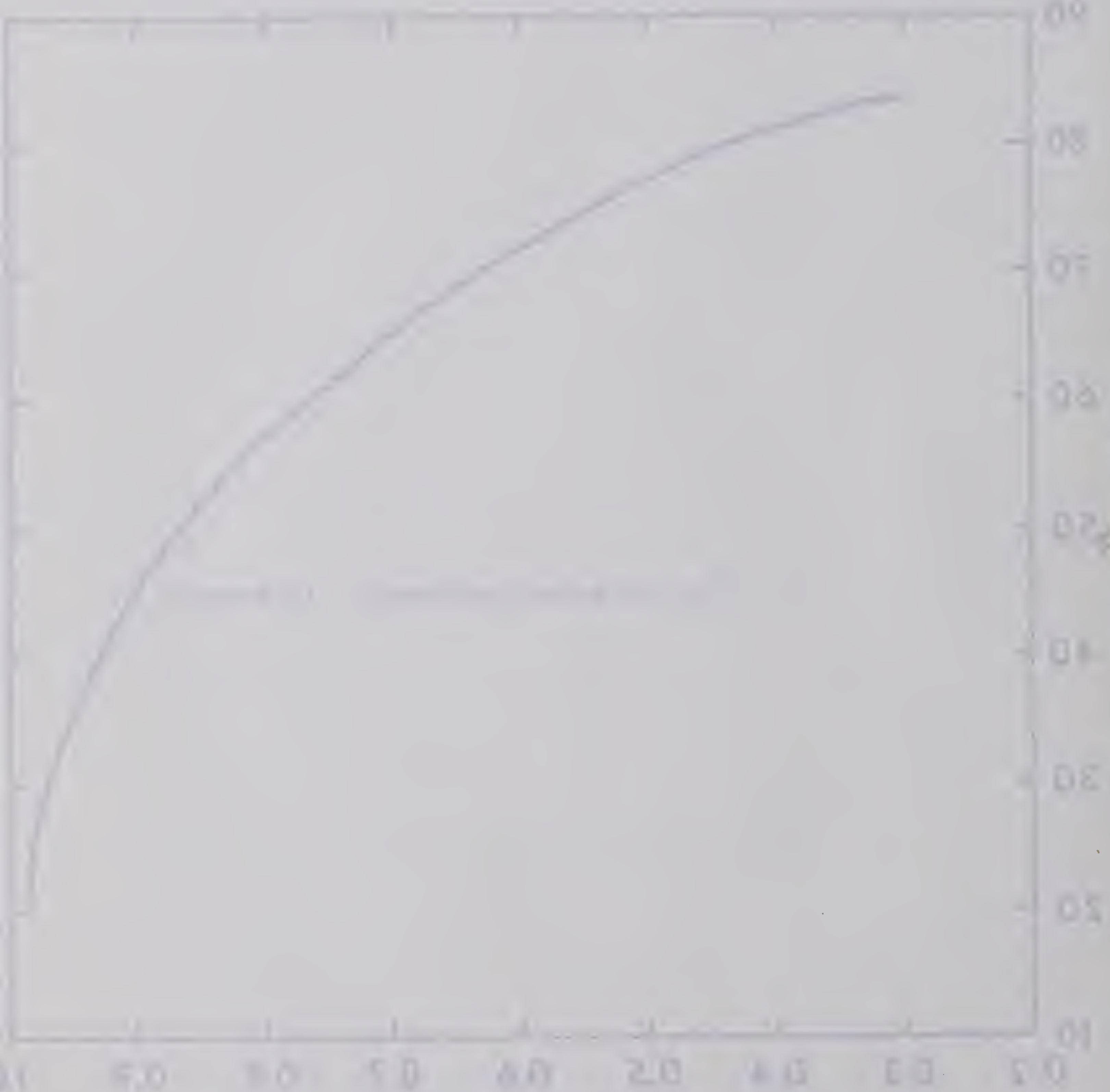
The butyl cellosolve was not included in the fluor solution used for plasma analysis since the plasma digest was immiscible with it. In this case the volume of butyl cellosolve in the above formula was replaced by an equal volume of toluene. No other modifications were made in the formula.

K. Radioassay of C^{14}-Salicylic Acid

The radioactivity of all samples was counted in the Nuclear Chicago 720 series liquid scintillation counter. This radioactivity gave a measure of the concentration of salicylic acid in each sample. The skin tissue, plasma, drug vehicle and digesting agents used produced varying degrees of quenching. Therefore all counts obtained were corrected to a theoretical disintegration per minute (DPM) using the standard channels ratio technique. This was done by first using the unquenched C^{14} standard to determine the appropriate channels ratio settings on the counter for C^{14}. A quenching correction curve of per cent efficiency versus the channels ratio was then plotted using C^{14} quenching standards (Figure 13). This curve was checked each time a series of samples was counted. The per cent efficiency with which each sample was counted was calculated from this curve and the sample counts were corrected accordingly. All three types of samples were counted at 50 per cent efficiency or better. Ten minute background counts were made on samples of the drug preparation, skin tissues and plasma. These background samples were identical to the samples prepared in experiments on each respective medium with the exception that the radioactive

Figure 13: Quenching curve for C^{14}.


90
80
70
60
50
40
30
20
10
% EFFICIENCY
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
CHANNEL RATIOS



drug was omitted. The background samples were counted and corrected for quenching each time a series of samples which they represented was counted. The corrected background counts were then subtracted from the respective corrected sample counts to give the theoretical DPM of the sample. All samples were counted for 10 minutes or until 10,000 counts had been recorded with the exception of the drug preparation uniformity samples which were counted at a pre-set count of 1,000,000.

An additional correction factor was applied to the DPM of plasma samples. The DPM of these samples represented the specific activity of salicylic acid in only 0.5 ml of plasma. Such specific activities would not be expected to represent the relative degrees of systemic absorption among animals which have different plasma volumes. The work of Ansill (108) and Masouredis and Melcher (109) indicated that the plasma volumes of guinea pigs vary according to the weight of the animal. Ansill reported that the combined results of the two groups gave a 3.9 ml per 100 Gm body weight value for the average plasma volume of male guinea pigs. The average weight of the animals used in the present study was 665 Gm. On the above basis, the average plasma volumes of animals used would be 25.9 ml. Therefore, to correct for the effect of the systemic dilution of salicylic acid due to varying plasma volumes, the DPM of each plasma sample was multiplied by the following factor:

$$\frac{\text{calculated plasma volume of test animal (ml)}}{25.9}$$

L. Autoradiography Procedure

Autoradiography was used in this study to confirm the presence of radioactive salicylic acid in the various regions of bald skin and not to quantitatively measure the amount of drug absorbed. The only difference in obtaining sections of a treated bald skin punch for autoradiography from obtaining sections of the same type of punch for scintillation analysis was in the method of slicing the respective punches. While the punch for scintillation analysis was sliced in 50 micron sections parallel to the epidermal surface, the punch for autoradiography was sliced in 30 micron sections perpendicular to the epidermal surface. In the latter procedure, an anti-roll device was used to unfold each section as it was sliced. Each 30 micron section was transferred from the microtome blade to prepared emulsion plates with a surgical dissecting needle. The emulsion plates used were one inch by three inch NTB-3 Nuclear plates manufactured by Eastman Kodak. While the plates were stored over Drierite* in a light-proof box at approximately -5°C, they were warmed to room temperature immediately prior to use. After the tissue sections were deposited on the emulsion side, the plate was returned to the light-proof box over Drierite for at least 24 hours to remove moisture from the tissue sections. Then, a one inch by three inch siliconized glass slide was carefully superimposed over the sections on the emulsion slide. These two slides were held together with an Esterbrook #2 clamp. This assembly was then placed once again in the light-proof box over Drierite and allowed to stand for

approximately two weeks at -5°C.

After exposure the siliconized glass slide was removed. Precautions were taken to do this before the slides became warm so that the tissue sections would not stick to the siliconized slide. The emulsion slide with the tissue was placed in Kodak D-19 developer for approximately four minutes. It was then washed in distilled water for 30 seconds before placing in Kodak Rapid Fixer for twice the length of time required to clear the emulsion. The slide with the sections was then washed in running distilled water for approximately 10 minutes. All solutions used were kept at 19 to 21°C, and all procedures involving the exposure of the emulsion slides were performed under controlled light conditions using a Wratten series #2 filter.

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* Drierite is anhydrous $CaSO_4$ manufactured by the W.A. Hammond Drierite Co., Xenia, Ohio, U.S.A.

RESULTS AND DISCUSSION

A. Developing the Method

Experimental variables affecting the uniform application of a specific amount of drug preparation to a specific surface area of skin were anticipated and methods for the control of these variables have been discussed previously (see pp. 43 to 47). The technical problems influencing the subsequent recovery of corresponding skin samples among replicates, however were determined only through extensive experimentation.

In the initial work, the measurement of intracutaneous absorption was carried out by applying the drug preparation to the skin of the animal on the dorsal surface after shaving. The skin was then sectioned at successively increasing depths from the outer surface. This recovery was effected by slicing from the epidermis toward the subcutaneous region (method I, see p. 48). A 10 per cent (18 μc/Gm) preparation of C^{14}-labeled salicylic acid (preparation A) was used. In the first experiment this preparation was applied by rubbing for 60 seconds. It was then left in contact with the skin for 120 minutes. The resulting amounts of radioactive drug at various depths in the skin of replicates receiving this treatment were analyzed and the values are presented in Table I. A high concentration level at each depth indicated significant absorption of salicylic acid by the skin. There appeared to be very little absorption by the deeper dermal layers and therefore only the concentration in the 250 microns of tissue

TABLE I

INTRACUTANEOUS ABSORPTION OF C^{14}-LABELED SALICYLIC ACID

METHOD I - (See Legend)

Skin Depth from Surface in Microns (μ)	DPM in 50 Micron Sections of Skin Replicates						Average DPM
	#1	#2	#3	#4	#5	#6	
100	100,553	76,594	128,389	76,594	99,303	126,082	101,252
150	55,089	54,594	59,187	45,642	51,927	61,081	54,587
200	28,291	20,578	29,018	17,654	19,556	25,501	23,433
250	11,958	9,990	13,603	6,883	6,162	6,668	9,211
300	4,833	5,226	4,879	3,433	3,031	2,248	3,942
350	3,548	1,436	1,942	1,463	1,244	551	1,697
Total Activity*	103,719	91,824	108,629	75,075	81,920	96,049	

AVERAGE TOTAL ACTIVITY* = 92,869 ± 12, 758**

Legend

1. Drug vehicle - Modulan-Olive Oil
2. Rubbing time - 60 seconds
3. Rest time - 120 minutes
4. Skin sample diameter - 19 mm
5. Skin type - hairy
6. Direction of slicing - epid. to subcut.
7. Drug concentration in vehicle - 10%
8. Specific activity of drug in vehicle - 18 μc/Gm

* Excludes first 100 μ which contain contamination from surface

** Standard Deviation

proximal to the epidermis was taken to represent the absorption by each replicate.

The effect of rubbing time on absorption was determined in the next experiment by reducing the rubbing time from 60 seconds to 10 seconds. A significant decrease in absorption (Table II) indicated that the degree of rubbing was an important factor in intracutaneous absorption. The effect of resting time was evaluated in a similar manner in experiment three by rubbing for 60 seconds but eliminating the rest time. Again, a decrease in absorption (Table III) from that found in the first experiment suggested that the duration of drug contact to the surface of the skin was also an influential factor in the process of absorption. Although the results of experiment three showed a decrease in absorption with a decrease in rest time, the magnitude of this decrease was not as large as could be expected. It appeared questionable whether the 60 second rubbing alone could produce the rapid absorption of drug by the deeper tissues which was suggested by the results. Suspicions were aroused that the drug preparation was being forced mechanically down the pilosebaceous route. Such mechanical induction probably would result in the high levels of radioactivity measured at each depth of skin but it would not be representative of true drug absorption. It was recognized that the potential problem of mechanical transfer of this nature could be avoided if skin which did not have pilosebaceous apparatus but which possessed the normal barriers to absorption could be obtained.

TABLE II

INTRACUTANEOUS ABSORPTION OF C^{14}-LABELED SALICYLIC ACID

METHOD I - REDUCED RUBBING TIME (See Legend)

Skin Depth from Surface in Microns (μ)	DPM in 50 Micron Sections of Skin Replicates						Average DPM
	#1	#2	#3	#4	#5	#6	
100	49,617	42,150	35,706	38,261	48,679	62,329	46,124
150	19,169	17,072	14,214	18,751	13,468	28,447	18,520
200	6,058	6,773	5,804	8,270	3,799	11,519	7,037
250	1,643	2,245	1,479	3,873	1,998	5,664	2,817
300	858	1,440	2,001	2,186	1,703	1,318	1,584
350	296	1,143	165	1,477	1,089	419	765
Total Activity*	28,024	28,673	23,663	34,557	22,057	47,367	

AVERAGE TOTAL ACTIVITY+ = 30,724 ± 8,101++

Legend

1. Drug vehicle - Modulan-Olive Oil
2. Rubbing time - 10 seconds
3. Rest time - 120 minutes
4. Skin sample diameter - 19 mm
5. Skin type - hairy
6. Direction of slicing - epid. to subcut.
7. Drug concentration in vehicle - 10%
8. Specific activity of drug in vehicle - 18 μc/Gm

* Excludes first 100 μ which contain contamination from surface

** Standard Deviation

TABLE III

INTRACUTANEOUS ABSORPTION OF C^{14}-LABELED SALICYLIC ACID

METHOD I - REDUCED REST TIME (See Legend)

Skin Depth from Surface in Microns (μ)	DPM in 50 Micron Sections of Skin Replicates						Average DPM
	#1	#2	#3	#4	#5	#6	
100	48,716	48,716	48,413	60,399	52,831	64,328	53,900
150	19,440	13,998	16,875	19,396	21,297	20,440	18,574
200	5,363	4,132	3,755	3,529	3,733	5,145	4,276
250	1,978	1,300	952	890	3,612	1,035	1,628
300	1,209	1,912	613	442	1,677	557	1,068
350	699	443	413	500	838	531	571
Total Activity*	28,689	21,785	22,608	24,757	31,157	27,708	

AVERAGE TOTAL ACTIVITY* = 26,117 ± 3,674**

Legend

1. Drug vehicle - Modulan-Olive Oil
2. Rubbing time - 60 seconds
3. Rest time - 0 minutes
4. Skin sample diameter - 19 mm
5. Skin type - hairy
6. Direction of slicing - epid. to subcut.
7. Drug concentration in vehicle - 10%
8. Specific activity of drug in vehicle - 18 μc/Gm

* Excludes first 100 μ which contain contamination from surface

** Standard Deviation

The bald area of the guinea pig was reported to be such a tissue. It was adopted for use in the present work to prevent the complication of trying to differentiate upon analysis the amount of drug absorbed by the skin from the amount transferred mechanically down the pilosebaceous route. Absorption studies on bald skin became by necessity studies on transepidermal absorption. Therefore results from such studies represented relative rather than absolute absorption since absorption occurring by only one route was measured. This was not, however, considered as a serious limitation of the "bald spot" as a model for intracutaneous absorption studies since the major objective of such studies would be to evaluate the relative effects which formulation factors might have upon intracutaneous absorption.

The use of the guinea pig "bald spots" was a significant second step taken to develop a method for measuring intracutaneous absorption. Skin sections from such treated sites were recovered for analysis by again slicing from the epidermis to the subcutaneous region (method II, see p. 52). Since the surface area of recovered bald skin specimens was only eight mm in diameter, 100 micron (2 x 50 μ) depths of skin were analyzed as samples from each replicate. This was done to achieve a satisfactory level of radioactivity in each sample for statistical counting purposes. The total depth of dermal tissue analyzed to represent absorption by each replicate was extended accordingly to 500 microns so that the drug distribution in each replicate again would be shown by five samples.

Experiment four was the first of a series of experiments planned to determine the effect on absorption of eliminating the pilosebaceous route. Preparation A was applied to the bald spots with 10 second rubbing and for zero rest time. The results of experiment four (Table IV) showed that although mechanically introduced drug may have interfered with absorption measurements in previous experiments, this artifact did not appear to be alone responsible for the high levels of drug analyzed at various depths in the skin. An autoradiograph (Figure 14) of a skin section

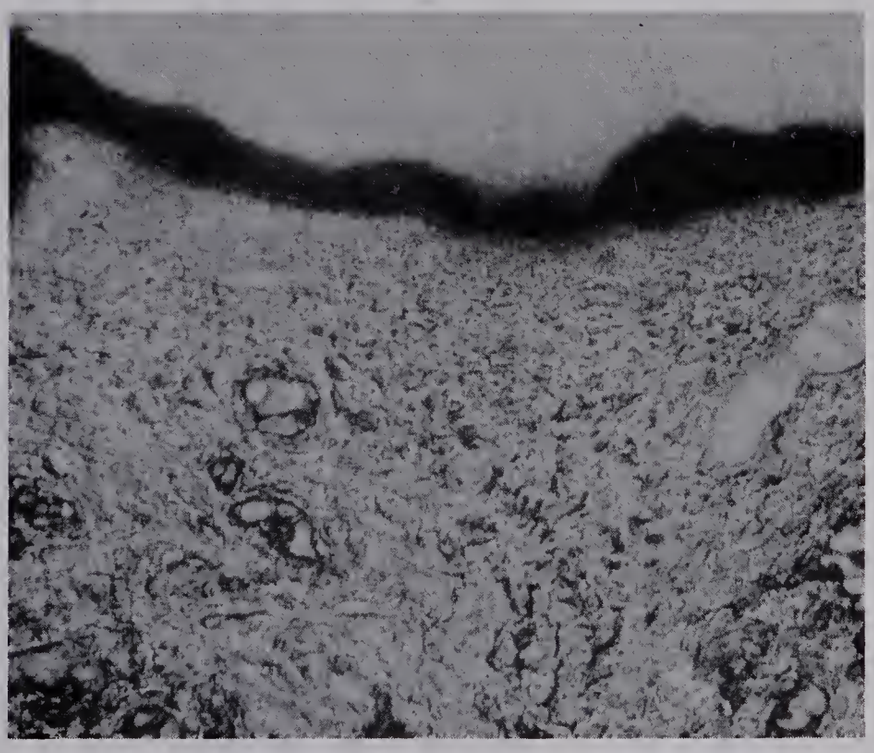


Figure 14: Autoradiograph of skin section showing depth of C^{14}-salicylic acid penetration from preparation A (x 40). (Section sliced from the subcutaneous region toward the epidermis).

TABLE IV

INTRACUTANEOUS ABSORPTION OF C^{14}-LABELED SALICYLIC ACID

METHOD II - (See Legend)

Skin Depth from Surface in Microns (μ)	DPM in 100 Micron Sections of Skin Replicates						Average DPM
	#1	#2	#3	#4	#5	#6	
100	25,980	33,456	23,440	27,279	19,308	17,193	24,443
200	14,593	18,402	19,787	15,833	14,589	13,945	16,192
300	9,145	8,803	9,264	7,651	10,510	10,414	9,298
400	4,012	2,891	4,415	3,866	4,427	2,191	3,634
500	2,262	2,920	1,272	1,255	738	575	1,504
600	512	315	34	472	42	154	255
Total Activity*	30,524	33,331	34,772	29,077	30,306	27,279	

AVERAGE TOTAL ACTIVITY* = 30,882 ± 2,751**

Legend

1. Drug vehicle - Modulan-Olive Oil
2. Rubbing time - 10 seconds
3. Rest time - 0 minutes
4. Skin sample diameter - 8 mm
5. Skin type - bald
6. Direction of slicing - epid. to subcut.
7. Drug concentration in vehicle - 10%
8. Specific activity of drug in vehicle - 18 μc/Gm

* Excludes first 100 μ which contain contamination from surface

** Standard Deviation

cut perpendicular to the surface from a tissue sample in experiment four supported the theory that some factor arising from the procedural technique probably was contributing to the absorption results which were being obtained. The autoradiograph showed no evidence of the presence of salicylic acid deeper than approximately 100 to 200 microns below the surface of the skin. This appeared to contradict the absorption results from similarly treated samples measured by scintillation counting. Since a different method of slicing was used in the recovery of skin sections for autoradiography than for scintillation analysis, the slicing procedure for the latter was considered responsible for producing erroneous absorption results.

By slicing skin sections parallel to the epidermal surface and in successive order toward the deeper tissues, the microtome knife would be expected to progress from a region of higher drug concentration to a region of lower drug concentration. Consequently, if the knife should transfer any drug from one section to the next, the drug concentrations in these sections would not be representative of absorption alone. Choman (44) pointed out that this situation could be prevented by freezing the skin sample to the specimen plate of the microtome with the epidermal surface down and then slicing from the subcutaneous surface toward the epidermis. Method III (see p. 52) represented such a slicing procedure. It was used in experiment five to determine whether contamination by the microtome knife contributed to the absorption results

obtained in experiment four.

The drug preparation, rubbing time and rest time used in experiment four were repeated for experiment five. The results shown in Table V supported the suspicion that the microtome knife was carrying contaminant into the successive sections which were sliced. Very little absorption was detected in tissues deeper than approximately 200 microns from the epidermal surface whereas results from experiment four (Table IV) showed considerable drug concentration as deep as 500 to 600 microns. The drug concentration gradients through the skin from experiments four and five are presented graphically in Figure 15. In view of these results, the data obtained in experiments one to four must be considered as not truly representing the intracutaneous absorption of salicylic acid. Furthermore, the validity of the values reported by Sheinaus, Christian and Sperandio (96) become open to question since the skin sections recovered for analysis in their work were sliced from the epidermis towards the subcutaneous fat. In the present study, attempts to slice shaved skin samples by method III were unsuccessful as the samples became detached from the specimen holder plate before sections near the epidermis could be recovered. This problem was attributed to the presence of residual hair on the surface of the sample which prevented the formation of a strong bond between the sample and the freezing plate. Consequently, the series of experiments designed to test the effect of the pilosebaceous route upon absorption was abandoned. Method III was then

TABLE V

INTRACUTANEOUS ABSORPTION OF C^{14}-LABELED SALICYLIC ACID

METHOD III - (See Legend)

Skin Depth from Surface in Microns (μ)	DPM in 100 Micron Sections of Skin Replicates						Average DPM
	#1	#2	#3	#4	#5	#6	
100	13,710	5,795	3,906	3,424	21,251	21,917	11,667
200	1,259	344	164	1,169	224	867	671
300	10	18	11	6	0	31	13
400	12	11	10	5	2	14	9
500	20	13	0	4	7	29	12
600	12	22	4	8	8	10	11
Total Activity*	1,313	408	189	1,192	241	951	

AVERAGE TOTAL ACTIVITY* = 716 ± 497**

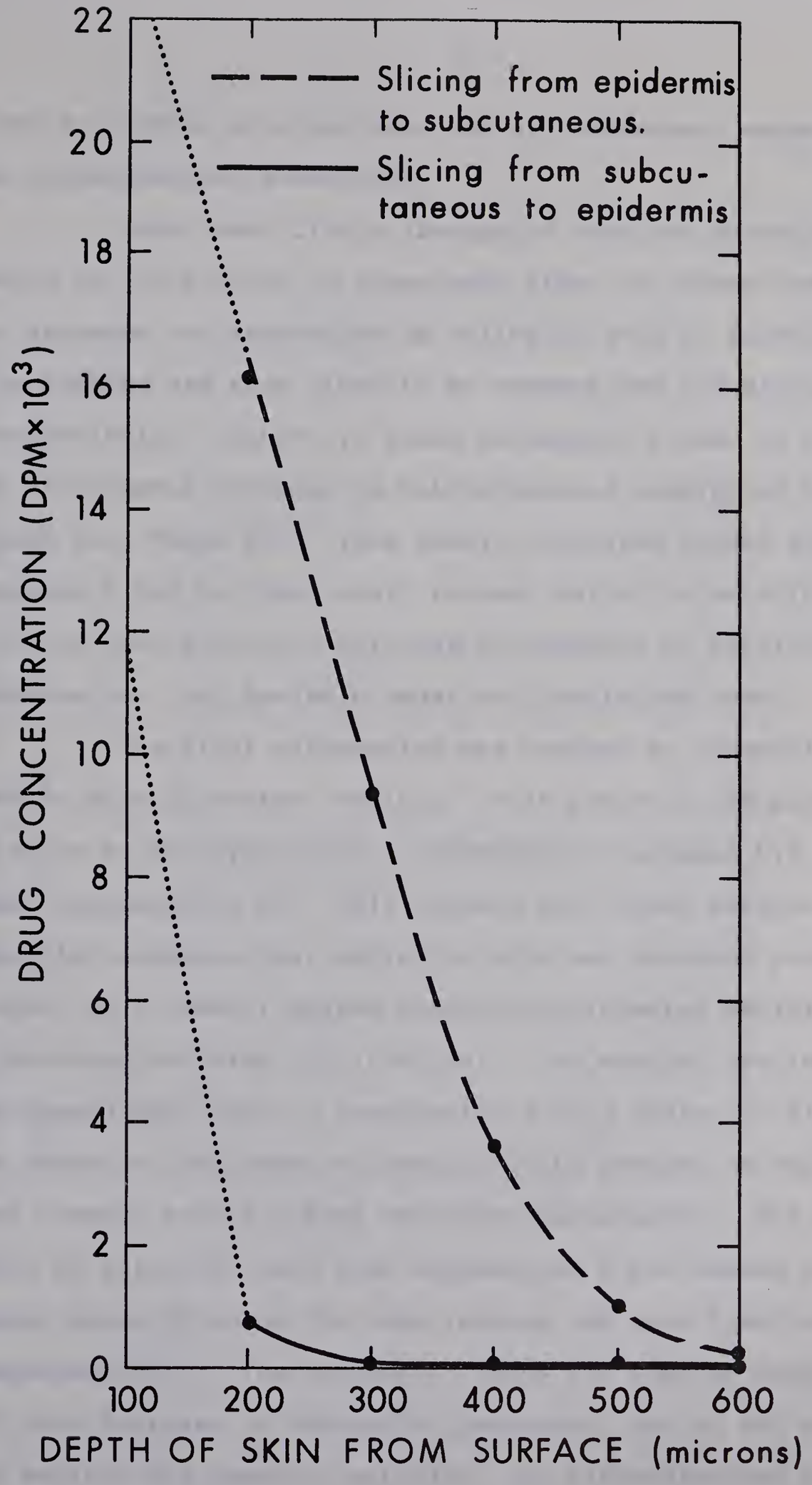
Legend

1. Drug vehicle - Modulan-Olive Oil
2. Rubbing time - 10 seconds
3. Rest time - 0 minutes
4. Skin sample diameter - 8 mm
5. Skin type - bald
6. Direction of slicing - subcut. to epid.
7. Drug concentration in vehicle - 10%
8. Specific activity of drug in vehicle - 18 μc/Gm

* Excludes first 100 μ which contain contamination from surface

** Standard Deviation

Figure 15: Drug concentration gradients in the skin obtained with different methods of slicing.


Slicing from epidermis to subcutaneous.
Slicing from subcutaneous to epidermis
DRUG CONCENTRATION (DPM×10³)
DEPTH OF SKIN FROM SURFACE (microns)
0
2
4
6
8
10
12
14
16
18
20
22
100
200
300
400
500
600

used to recover skin sections for all subsequent experiments on intracutaneous absorption.

Since very little absorption occurred beyond a depth of 200 microns in experiment five, an attempt was made to increase the absorption of salicylic acid by increasing the rubbing and rest times to 60 seconds and 240 minutes respectively. Changes in these parameters failed to produce an anticipated increase in intracutaneous absorption in experiment six (Table VI). This result indicated either that preparation A did not adequately release salicylic acid for absorption or that salicylic acid was not capable of significant penetration into the skin under the conditions used.

The first alternative was checked by preparing salicylic acid in another vehicle. A 10 per cent (90 μc/Gm) preparation of salicylic acid in Hydrophilic Ointment U.S.P. was made (preparation B), This vehicle was chosen because of reported evidence that salicylic acid was absorbed percutaneously to a greater degree from an oil-in-water emulsion than from other vehicles (52,70,87,98). The specific activity was increased over that in preparation A by a factor of five in an effort to increase the radioactivity present in the tissues and thereby enable a more sensitive measurement. The absorption of salicylic acid from preparation B was tested in experiment seven by using the same rubbing and rest times as in experiment six. The results in Table VII show an approximately 25 fold increase in absorption presumably due to the changes in vehicle and specific activity. An autoradiograph of a skin

TABLE VI

INTRACUTANEOUS ABSORPTION OF C^{14}-LABELED SALICYLIC ACID

METHOD III - INCREASED RUBBING & REST TIMES (See Legend)

Skin Depth from Surface in Microns (μ)	DPM in 100 Micron Sections of Skin Replicates						Average DPM
	#1	#2	#3	#4	#5	#6	
100	11,492	4,536	5,240	15,014	1,168	6,829	7,380
200	2,338	7	1,103	1,049	599	1,142	1,040
300	10	0	23	0	6	0	6
400	19	10	41	0	9	0	13
500	0	0	0	9	8	30	8
600	19	10	0	5	7	3	7
Total Activity*	2,386	27	1,167	1,063	629	1,175	

AVERAGE TOTAL ACTIVITY* = 1,074 ± 779**

Legend

1. Drug vehicle - Modulan-Olive Oil
2. Rubbing time - 60 seconds
3. Rest time - 240 minutes
4. Skin sample diameter - 8 mm
5. Skin type - bald
6. Direction of slicing - subcut. to epid.
7. Drug concentration in vehicle - 10%
8. Specific activity of drug in vehicle - 18 μc/Gm

* Excludes first 100 μ which contain contamination from surface

** Standard Deviation

TABLE VII

INTRACUTANEOUS ABSORPTION OF C^{14}-LABELED SALICYLIC ACID

METHOD III - NEW VEHICLE & SPECIFIC ACTIVITY (See Legend)

Skin Depth from Surface in Microns (μ)	DPM in 100 Micron Sections of Skin Replicates						Average DPM
	#1	#2	#3	#4	#5	#6	
100	84,121	88,799	51,846	37,119	106,577	24,001	65,410
200	7,552	6,794	8,023	9,422	7,595	9,053	8,073
300	5,485	5,671	7,338	8,599	6,577	5,431	6,517
400	3,420	4,894	6,171	6,906	5,568	4,816	5,296
500	3,104	4,242	5,489	5,815	4,220	4,104	4,496
600	2,773	3,538	3,555	4,714	2,430	3,416	3,404
Total Activity*	22,334	25,139	30,576	35,456	26,390	26,820	

AVERAGE TOTAL ACTIVITY* = 27,785 ± 4,609**

Legend

1. Drug vehicle - Hydrophilic Oint. USP XVII
2. Rubbing time - 60 seconds
3. Rest time - 240 minutes
4. Skin sample diameter - 8 mm
5. Skin type - bald
6. Direction of slicing - subcut. to epid.
7. Drug concentration in vehicle - 10%
8. Specific activity of drug in vehicle - 90 μc/Gm

* Excludes first 100 μ which contain contamination from surface

** Standard Deviation

sample from experiment seven confirmed the deep dermal absorption of salicylic acid from preparation B (Figure 16). Since the specific activity was increased by a factor of only five, much of the increased absorption had to be attributed to the new vehicle. Plasma measurements of salicylic acid from the two experiments similarly showed a relatively higher systemic absorption from preparation B than from preparation A (Table VIII). Since the degree of absorption from preparation B appeared to be adequate for the purpose of comparing relative absorption by the two tissues, no further investigation was attempted

Figure 16: Autoradiograph of skin section showing depth of C^{14}- salicylic acid penetration from preparation B (x 40). (Section sliced from the subcutaneous region toward the epidermis).

TABLE VIII

SYSTEMIC ABSORPTION OF C^{14}-LABELED SALICYLIC ACID (See Legend)

EFFECT OF VEHICLE AND SPECIFIC ACTIVITY

Vehicle Specific Activity	Replicate Numbers	Average DPM[a] in 0.5 ml Plasma Replicates #1	#2	#3
Modulan-Olive Oil 18 µc/Gm	DPM	0	0	4
	Correction Factor[b]	-	-	1.0
	Corrected DPM	0	0	4
AVERAGE DPM AMONG REPLICATES = 1				
Hydrophilic Oint. USP XVII 90 µc/Gm	DPM	3,940	5,212	6,497
	Correction Factor[b]	1.04	0.96	1.02
	Corrected DPM	4,098	5,004	6,627
AVERAGE DPM AMONG REPLICATES = 5,243 ± 1,281[c]				

Legend:

1. Drug concentration in vehicle = 10%
2. Rubbing time = 60 secs.
3. Rest time = 240 mins.

a. Average of duplicate determinations for each replicate.
b. Corrected to constant plasma volume among replicates (see p.58).
c. Standard Deviation.

to define the characteristics of the two vehicles which were responsible for the difference in absorption. However, such a study should prove to be valuable and is a possible future extension of the present work.

B. Percutaneous Absorption as a Function of Rest Time

The effect of different rest times upon intracutaneous and systemic absorption was investigated to compare the relative drug absorption by these two phases. Drug preparation B was used in this study for the reason presented above. A common rubbing time was required for each experiment to keep constant any effect which rubbing might have upon the degree of absorption. A minimum amount of rubbing was preferred for several reasons. First, minimum rubbing would approximate the degree of rubbing used in clinical application. Second, if the degree of rubbing should significantly influence absorption, then the effect of excessive rubbing might preclude the detection of the effect of the rest times being studied. Furthermore, excessive rubbing was observed to produce an erythema of the skin receiving application. This would indicate a dilatation of the blood vessels near the surface which could cause an abnormally high systemic absorption. Therefore, experiments were performed to determine the minimum rubbing time required to produce a significant concentration of drug absorbed throughout the skin.

The results of experiment seven showed the effect of 60 seconds rubbing after 240 minutes rest (Table VII). The contribution of this rubbing time to absorption was determined

in experiment eight by eliminating the rest period. The results as shown in Table IX indicated that 60 second rubbing had very little effect on intracutaneous absorption. This was verified later by experiment nine where the preparation was rubbed for 10 seconds with a 240 minute rest period. Table X showed that an almost equal amount of absorption resulted after 240 minutes rest with 10 second rubbing as occurred with 60 second rubbing and the same rest time (Table VII). Furthermore, 10 second rubbing with no rest period was found to promote very little absorption in experiment ten (Table XI). No further reduction in rubbing time was considered acceptable since it was noticed that rubbing for less than 10 seconds did not provide a uniform application of the drug preparation over the entire surface area of skin exposed.

Although rubbing time variations appeared to have little effect on intracutaneous absorption, plasma measurements from the above experiments showed a significant difference in the systemic absorption of salicylic acid due to rubbing time (Table XII). After 240 minutes of rest, the absorption obtained with 60 second rubbing was much greater than that obtained with 10 second rubbing. The effect of increased rubbing on the blood vessels near the surface of the skin as postulated earlier appears to be a logical explanation for this difference in absorption. Since the two rubbing times were found to promote intracutaneous absorption to approximately the same degree, but 60 second rubbing had a greater influence on systemic absorption than did 10 second rubbing, the latter

TABLE IX

INTRACUTANEOUS ABSORPTION OF C^{14}-LABELED SALICYLIC ACID

EFFECT OF RUBBING & REST TIMES A. (See Legend)

Skin Depth from Surface in Microns (μ)	DPM in 100 Micron Sections of Skin Replicates						Average DPM
	#1	#2	#3	#4	#5	#6	
100	133,246	76,913	5,225	3,565	11,695	4,464	39,185
200	93	77	113	52	170	61	94
300	27	64	9	66	1	3	28
400	33	28	11	0	114	7	32
500	27	48	69	8	87	0	40
600	15	53	0	8	95	81	42
Total Activity*	195	270	202	134	467	152	

AVERAGE TOTAL ACTIVITY* = 237 ± 122**

Legend

1. Drug vehicle - Hydrophilic Oint. USP XVII
2. Rubbing time - 60 seconds
3. Rest time - 0 minutes
4. Skin sample diameter - 8 mm
5. Skin type - bald
6. Direction of slicing - subcut. to epid.
7. Drug concentration in vehicle - 10%
8. Specific activity of drug in vehicle - 90 μc/Gm

* Excludes first 100 μ which contain contamination from surface

** Standard Deviation

TABLE X

INTRACUTANEOUS ABSORPTION OF C^{14}-LABELED SALICYLIC ACID

EFFECT OF RUBBING & REST TIMES B. (See Legend)

Skin Depth from Surface in Microns (μ)	DPM in 100 Micron Sections of Skin Replicates						Average DPM
	#1	#2	#3	#4	#5	#6	
100	133,247	119,199	177,690	59,173	47,266	66,163	100,456
200	6,439	6,737	5,669	4,929	6,758	7,271	6,300
300	5,981	5,822	4,819	4,514	5,703	6,430	5,545
400	5,508	4,945	4,495	4,223	4,207	5,575	4,826
500	3,212	3,445	4,121	3,663	4,246	4,176	3,810
600	2,450	2,413	3,519	2,996	2,950	2,873	2,867
Total Activity*	23,590	23,362	22,623	20,325	23,864	26,325	

AVERAGE TOTAL ACTIVITY* = 23,348 ± 1,941**

Legend

1. Drug vehicle - Hydrophilic Oint. USP XVII
2. Rubbing time - 10 seconds
3. Rest time - 240 minutes
4. Skin sample diameter - 8 mm
5. Skin type - bald
6. Direction of slicing - subcut. to epid.
7. Drug concentration in vehicle - 10%
8. Specific activity of drug in vehicle - 90 μc/Gm

* Excludes first 100 μ which contain contamination from surface

** Standard Deviation

TABLE XI

INTRACUTANEOUS ABSORPTION OF C^{14}-LABELED SALICYLIC ACID

EFFECT OF RUBBING & REST TIMES C. (See Legend)

Skin Depth from Surface in Microns (μ)	DPM in 100 Micron Sections of Skin Replicates						Average DPM
	#1	#2	#3	#4	#5	#6	
100	53,235	17,955	37,055	83,596	34,563	31,088	42,915
200	119	79	120	71	34	45	78
300	96	2	104	54	23	0	46
400	75	0	119	65	0	33	48
500	0	1	29	1	0	29	10
600	9	32	53	8	14	20	23
Total Activity*	299	114	425	199	71	127	

AVERAGE TOTAL ACTIVITY* = 206 ± 134**

Legend

1. Drug vehicle - Hydrophilic Oint. USP XVII
2. Rubbing time - 10 seconds
3. Rest time - 0 minutes
4. Skin sample diameter - 8 mm
5. Skin type - bald
6. Direction of slicing - subcut. to epid.
7. Drug concentration in vehicle - 10%
8. Specific activity of drug in vehicle - 90 μc/Gm

* Excludes first 100 μ which contain contamination from surface

** Standard Deviation

TABLE XII

SYSTEMIC ABSORPTION OF C^{14}-LABELED SALICYLIC ACID (See Legend)

EFFECT OF RUBBING AND REST TIMES

Average DPM[a] in 0.5 ml Plasma Replicates

Rubbing Time (sec.)	Rest Time (mins.)	240			0		
	Replicate No.	#1	#2	#3	#1	#2	#3
60	DPM	3,940	5,212	6,497	57	69	43
	Correction Factor[b]	1.04	0.96	1.02	1.02	0.96	1.02
	Corrected DPM	4,098	5,004	6,627	58	66	44
		AVERAGE DPM AMONG REPLICATES = 5,243 ± 1,281[c]			AVERAGE DPM AMONG REPLICATES= 56 ± 11[c]		
10	DPM	1,758	2,326	2,784	30	1	1
	Correction Factor[b]	1.08	1.04	0.99	1.08	0.95	0.95
	Corrected DPM	1,899	2,419	2,756	32	1	1
		AVERAGE DPM AMONG REPLICATES = 2,358 ± 438[c]			AVERAGE DPM AMONG REPLICATES = 11 ± 18[c]		

Legend:

1. Drug vehicle - Hydrophilic Oint. USP XVII
2. Drug concentration in vehicle - 10%
3. Specific activity - 90 μc/Gm

a. Average of duplicate determinations for each replicate.
b. Corrected to constant plasma volume among replicates (see p. 58).
c. Standard Deviation.

was decided upon for use in the "rest time" study.

The number of replicates used for each experiment was largely decided by the significance of the results obtained for each factor investigated. Although three plasma replicates appeared to be adequate for experiments six to 10, the number of such replicates was increased to six for studying rest time and concentration as functions of systemic absorption to equate with the number of replicates measured for intracutaneous absorption. This was considered desirable since the results from such studies would be used to interpret whether systemic and intracutaneous absorption were proportional.

The rest timesinvestigated were 240, 120 and 30 minutes. The results in Tables X, XIII and XIV show that the rate of intracutaneous absorption of salicylic acid changes with the times studied. This change in rate is probably best demonstrated in Figure 17 where the absorption at 240 minutes rest time was taken to represent 100 per cent absorption. While only about eight per cent of drug was absorbed during the first 12.5 per cent of rest time (30 minutes), absorption was approximately 67 per cent complete at 50 per cent of the rest period. Table XV shows that the rate of systemic absorption also changed at the different rest times. The trend in this change, however, differed from that found in intracutaneous absorption (Figure 17). Approximately two per cent of the drug was absorbed systemically during the first 12.5 per cent of time and absorption was

TABLE XIII

INTRACUTANEOUS ABSORPTION OF C^{14}-LABELED SALICYLIC ACID

EFFECT OF REST TIME A. (See Legend)

Skin Depth from Surface in Microns (μ)	DPM in 100 Micron Sections of Skin Replicates						Average DPM
	#1	#2	#3	#4	#5	#6	
100	93,432	106,570	192,404	48,692	94,609	106,570	107,461
200	4,952	4,795	3,569	3,988	5,804	5,403	4,752
300	4,552	4,030	2,984	3,109	4,730	4,447	3,975
400	3,873	3,331	2,536	2,454	3,704	3,433	3,222
500	2,756	2,254	1,875	1,807	2,462	2,500	2,276
600	1,445	1,391	1,478	1,359	1,486	1,421	1,430
Total Activity*	17,578	15,801	12,442	12,717	18,186	17,204	

AVERAGE TOTAL ACTIVITY* = 15,655 ± 2,505**

Legend

1. Drug vehicle - Hydrophilic Oint. USP XVII
2. Rubbing time - 10 seconds
3. Rest time - 120 minutes
4. Skin sample diameter - 8 mm
5. Skin type - bald
6. Direction of slicing - subcut. to epid.
7. Drug concentration in vehicle - 10%
8. Specific activity of drug in vehicle - 90 μc/Gm

* Excludes first 100 μ which contain contamination from surface

** Standard Deviation

TABLE XIV

INTRACUTANEOUS ABSORPTION OF C^{14}-LABELED SALICYLIC ACID

EFFECT OF REST TIME B. (See Legend)

Skin Depth from Surface in Microns (μ)	DPM in 100 Micron Sections of Skin Replicates						Average DPM
	#1	#2	#3	#4	#5	#6	
100	122,210	75,616	16,791	66,568	31,077	72,628	64,148
200	717	814	719	942	1,092	632	819
300	480	504	636	704	767	566	610
400	220	201	424	314	356	118	272
500	126	165	277	202	184	2	159
600	88	21	152	122	87	0	78
Total Activity*	1,631	1,705	2,208	2,284	2,486	1,318	

AVERAGE TOTAL ACTIVITY* = 1,939 ± 453**

Legend

1. Drug vehicle - Hydrophilic Oint. USP XVII
2. Rubbing time - 10 seconds
3. Rest time - 30 minutes
4. Skin sample diameter - 8 mm
5. Skin type - bald
6. Direction of slicing - subcut. to epid.
7. Drug concentration in vehicle - 10%
8. Specific activity of drug in vehicle - 90 μc/Gm

* Excludes first 100 μ which contain contamination from surface

** Standard Deviation

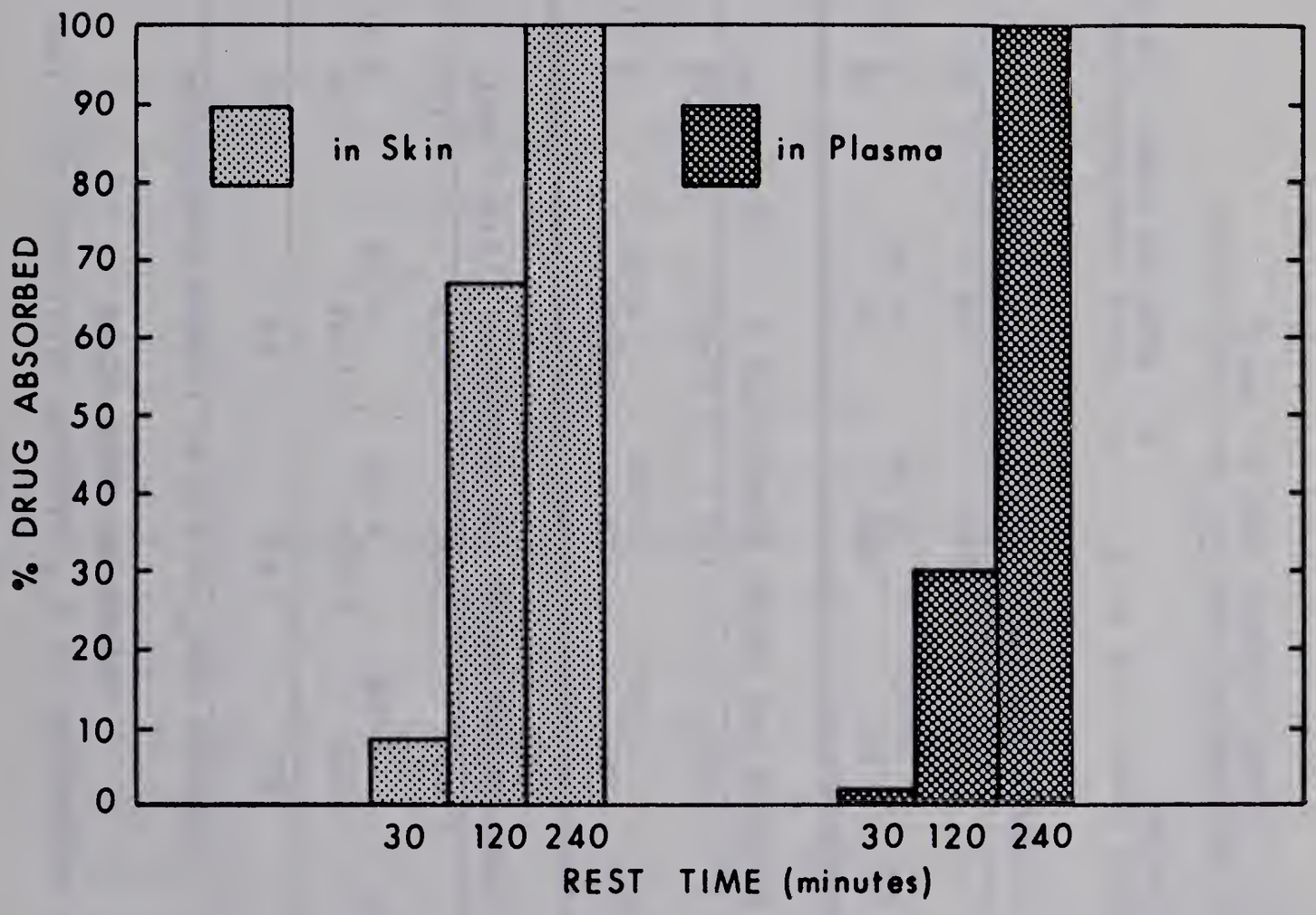


Figure 17: The absorption of C^{14}-labeled salicylic acid by skin and by plasma as a function of rest time.

TABLE XV

SYSTEMIC ABSORPTION OF C^{14}-LABELED SALICYLIC ACID (See Legend)

EFFECT OF REST TIMES

Rest Time (Mins.)	Replicate Numbers	Average DPM[a] in 0.5 ml Plasma Replicates #1	#2	#3	#4	#5	#6
240	DPM	1,758	2,326	2,784	4,238	3,636	4,136
	Correction Factor[b]	1.08	1.04	0.99	0.97	0.95	0.98
	Corrected DPM	1,899	2,419	2,756	4,111	3,454	4,053
	AVERAGE DPM AMONG REPLICATES = 3,115 ± 903[c]						
120	DPM	680	728	1,517	756	894	1,055
	Correction Factor[b]	1.08	1.02	0.95	1.01	0.99	0.95
	Corrected DPM	734	742	1,441	764	885	1,002
	AVERAGE DPM AMONG REPLICATES = 928 ± 272[c]						
30	DPM	33	199	51	25	109	43
	Correction Factor[b]	1.11	0.94	0.95	0.97	0.98	0.99
	Corrected DPM	37	187	48	24	107	42
	AVERAGE DPM AMONG REPLICATES = 74 ± 62[c]						

Legend:

1. Drug vehicle - Hydrophilic Oint. USP XVII
2. Drug concentration in vehicle - 10%
3. Specific activity - 90 μc/Gm
4. Rubbing time - 10 secs.

a. Average of duplicate determinations for each replicate.
b. Corrected to constant plasma volume (see p. 58).
c. Standard Deviation.

only about 30 per cent complete when half the rest period was finished. This trend in the systemic absorption of salicylic acid from a hydrophilic ointment at different rest times agreed quite closely with that found by Stolar, Rossi and Barr (70). The difference in the rates of intracutaneous and systemic absorption demonstrated that absorption by the two phases was not proportional at different rest times under the conditions of the present investigation. The reasons for this difference in absorption trends probably can be postulated better by first considering from a theoretical point of view what effect rest time might be expected to have upon absorption.

Higuchi (37) formulated two relationships of absorption as a function of time. The relationship which would be applicable to a particular system would depend on where the rate determining step was located in absorption from the system. Since the salicylic acid was present in preparation B principally in the suspended form the rate determining step to absorption in this study was expected to be in the vehicle (see p. 18). Therefore it was anticipated that equation 4 would predict the degree of absorption from preparation B better than would equation 2. The integrated form of equation 4 shows that absorption should vary according to the square root of time.

$$Q = \sqrt{2ADC_s t} \qquad \text{(Eq. 5)}$$

Since the variables A, D and C_s were kept constant for the "rest time" experiments, equation 5 may be reduced to

$$Q = \sqrt{X \cdot t} \qquad \text{(Eq. 6)}$$

where:

Q = concentration of drug absorbed (DPM)

X = a constant

t = rest time (minutes)

Theoretical Q values were calculated from equation 6 for intracutaneous and systemic absorption at the rest times studied in the following manner. Values for the constant (X) were calculated for both the skin (X_s) and plasma (X_p) phases by letting the experimental absorption results of both phases from the 240 minute rest period study represent Q_s and Q_p respectively.

This rest time was chosen to calculate the values for X since absorption at 240 minutes was expected to more closely represent that predicted by equation 6 than was absorption at a shorter time. The reason for this is that equation 6 was designed with the qualification that it would only predict absorption by a perfect sink. Absorption by the plasma and by the dermal tissues, however, would not be expected to initially approximate such a receptor. A certain lag time probably would occur after application of the drug preparation before the drug could penetrate the superficial tissues and appear in the blood and the dermis for analysis. Once the rate of drug penetration through the superficial tissues was constant, this lag time would be over and then the blood and/or the dermis might approximate a perfect sink for subsequent drug absorption. At this time, absorption by either or both phases could be expected to correspond to the absorption predicted by equation 6.

Therefore, absorption results obtained at the longest rest period studied, 240 minutes, were used for calculating X_s and X_p.

The 240 minute data from Table X was used to calculate a value for X_s as follows:

$$Q_s = \sqrt{X_s \cdot t}$$

$$23,348 = \sqrt{X_s \cdot 240}$$

$$X_s = 2,268,036$$

Systemic absorption results at 240 minutes from Table XV were similarly used to calculate a value for X_p

$$Q_p = \sqrt{X_p \cdot t}$$

$$3,115 = \sqrt{X_p \cdot 240}$$

$$X_p = 40,401$$

The predicted Q values for absorption by each phase were then calculated for 30 and 120 minute rest times by applying the above values for X_s and X_p. The predicted and corresponding experimental values for absorption at different rest times are presented in Table XVI.

TABLE XVI

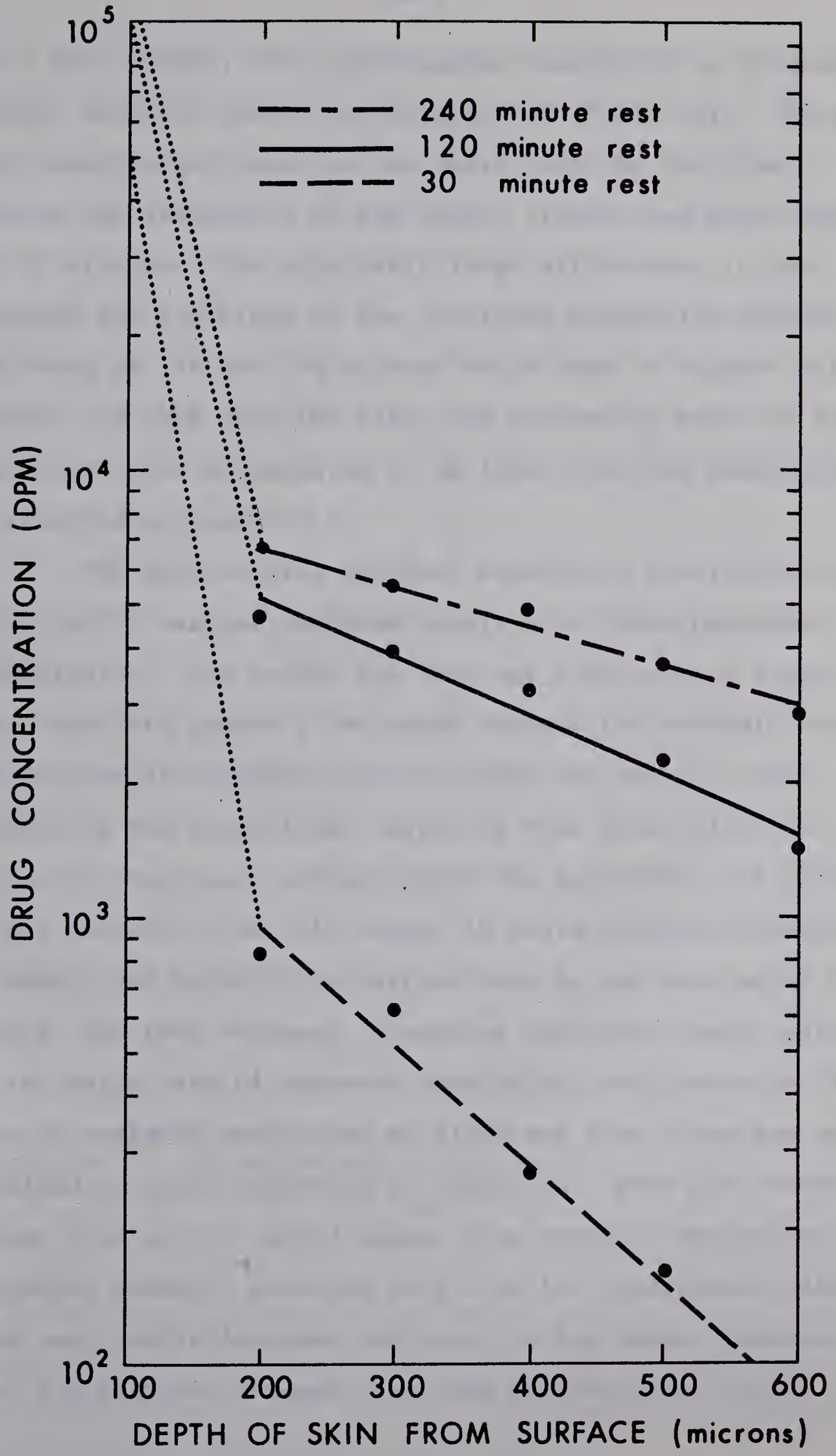
COMPARISON OF THEORETICAL (Eq. 6) AND EXPERIMENTAL ABSORPTION (DPM) of C^{14}-LABELED SALICYLIC ACID BY SKIN AND PLASMA AT DIFFERENT REST TIMES

	ABSORPTION (DPM)			
	SKIN		PLASMA	
Time (Mins.)	Theor.	Exptl.	Theor.	Exptl.
240*	23,348	23,348	3,115	3,115
120	16,497	15,655	2,202	928
30	8,249	1,939	1,101	74

* Theoretical absorption at this rest time arbitrarily was taken to be the same as that obtained experimentally.

The intracutaneous absorption determined experimentally at 120 minutes agreed very closely with that predicted. The experimental concentration gradients of drug through the skin at various rest times (Figure 18) show that the intercepts of the 120 and 240 minute slopes are fairly close to each other at the 200 micron level of skin. This indicates that the amount of drug entering the dermal tissues was nearly the same and, therefore, that the lag time was almost over at these rest times. Since the dermal tissues would be expected to function as a perfect sink after the completion of lag time, the absorption determined experimentally should approximate that predicted. This appeared to be the case for the 120

Figure 18: Concentration gradients of C^{14}-labeled salicylic acid in the skin at different rest times.


240 minute rest
120 minute rest
30 minute rest
DRUG CONCENTRATION (DPM)
10^5
10^4
10^3
10^2
100
200
300
400
500
600
DEPTH OF SKIN FROM SURFACE (microns)

minute rest period. The experimental absorption at 30 minutes however, was much lower than anticipated (Table XVI). This again could be explained on the basis that the lag time required for absorption by the dermal tissues was much longer than 30 minutes. The relatively large differences in the intercept and the slope of the 30 minute absorption gradient from those at 120 and 240 minutes would seem to support this concept. If such were the case, the absorption measured at 30 minutes would be expected to be lower than the absorption as predicted by equation 6.

The experimental systemic absorption results obtained at 120 and 30 minutes compared poorly with those predicted theoretically. The reason for this was difficult to explain since there are probably two major sources for systemic absorption located at different depths within the skin. A drug penetrating the superficial layers of skin would first encounter the capillary network below the epidermis. If it were not all absorbed from this region it would progress through the dermis and probably be carried away by the next major blood network, the rete cutaneum. Assuming that this deeper network was the major site of systemic absorption, the change in the rates of systemic absorption at different rest times may be explained by again referring to Figure 18. From the concentration gradients it would appear that systemic absorption at 30 minutes probably occurred only from the superficial network since very little drug was indicated in the deeper tissues. After 120 minutes, however, the drug concentration in the

deeper tissues increased rapidly and some systemic absorption probably occurred from the major vascular network as well. Since the concentration of drug in the deeper tissues apparently continued to increase with rest time, the amount of drug exposed to the rete cutaneum would therefore be expected to increase proportionally. Consequently, the rate of systemic absorption at 240 minutes was found to be faster than at 120 minutes. This change in rate of systemic absorption at the rest times studied suggested that the lag time required for systemic absorption to follow the absorption predicted by equation 6 was not realized before 240 minutes. On this basis, the experimental and theoretical systemic absorption data in Table XVI would be expected to differ accordingly.

Another relationship of absorption with time was proposed by Higuchi (37) with the assumption that the rate determining step to absorption occurred in the "major barrier" of the skin. Upon integrating the equation expressing this relationship (see equation 2, p. 16) the predicted absorption becomes directly proportional to time

$$Q = \frac{a}{\gamma} \frac{DA}{L} \cdot t \qquad \text{(Eq. 7)}$$

A comparison of the experimental intracutaneous and systemic absorption results with absorption predicted by this equation was made to see if the rate determining step to absorption in the present study was across the "major barrier". The theoretical values for absorption were calculated in the same manner as for equation 6 (see p. 90) and are compared to experimental

values in Table XVII.

TABLE XVII

COMPARISON OF THEORETICAL (Eq. 7) AND EXPERIMENTAL ABSORPTION (DPM) OF C^{14}-LABELED SALICYLIC ACID BY SKIN AND PLASMA AT DIFFERENT REST TIMES

Time (mins.)	ABSORPTION (DPM)			
	SKIN		PLASMA	
	Theor.	Exptl.	Theor.	Exptl.
240*	23,348	23,348	3,115	3,115
120	11,640	15,655	1,560	928
30	2,910	1,939	390	74

* Theoretical absorption at this rest time was arbitrarily taken to be the same as obtained experimentally.

The experimental absorption results of both skin and plasma differed considerably from the absorption values predicted by equation 7 for 120 and 30 minute rest times. Thus it appeared that the "major barrier" in these experiments was not the rate determining step to absorption as interpreted by equation 7.

The above interpretations of differences in the intracutaneous and systemic absorption of salicylic acid are presented but certain limitations are recognized. The use of plasma analysis to represent the amount of drug which is absorbed systemically is subject to variables such as metabolism, storage and excretion of the drug. The assumption that the rete cutaneum was the major source of systemic absorption would have been stronger if the analysis of dermal tissues

had been extended to a depth at which this vascular network is located. Intracutaneous absorption as measured by the described method was interpreted to have agreed favorably with the absorption predicted by equation 6 on the basis that the lag time was almost over after 120 minutes. This could have been further substantiated by performing absorption studies at progressively increasing rest times. The basic purpose of this study, however, was to determine whether systemic absorption was proportional to intracutaneous absorption. Since this purpose appeared to be satisfied with the data obtained, further investigations were not undertaken.

C. Percutaneous Absorption as a Function of Concentration

The primary object of studying concentration was to test the ability of the developed method to measure the effect of this variable on intracutaneous absorption. A comparison of theoretical absorption values with those obtained experimentally was adopted as a suitable means for making such an evaluation.

The theoretical absorption values for the five and 10 per cent concentrations studied were calculated by using equation 5. This equation was selected since it was found to be satisfactory for predicting the effect of rest time on the intracutaneous absorption of salicylic acid after allowing for duration of lag time. A 240 minute rest period was employed since results from the previous study also indicated that the tissues being measured best approximated a perfect sink at this time.

Two types of drug concentrations are described by equation 5. These are the concentration of dissolved drug (C_s) and the total concentration of both dissolved and suspended drug (A). Since salicylic acid was found to be present in the suspended form at both five and 10 per cent concentrations in the hydrophilic ointment, it was assumed that the amount of drug in the dissolved form (C_s) was constant for both preparations used in this investigation. On this basis, the only concentration variable of interest would be A and thus equation 5 may be reduced to

$$Q = \sqrt{X \cdot A} \qquad \text{(Eq. 8)}$$

The concentration of the 10 per cent preparation was arbitrarily taken to be exactly 100.0 mg/Gm. Since the five per cent preparation was made from the 10 per cent one, it's concentration was calculated from the relative specific activities of the two preparations and found to be 50.8 mg/Gm (see p. 54). The theoretical value of X was determined as previously by letting the experimental absorption from the five per cent preparation represent the theoretical absorption at this concentration. The predicted absorption value for the 10 per cent preparation was then calculated with this value of X.

The experimental absorption results for the five and 10 per cent concentrations of salicylic acid are shown in Table XVIII and X respectively. A comparison of the predicted (Eq. 8) and experimentally determined absorption values for the 10 per cent preparation is presented in Table XIX.

TABLE XVIII

INTRACUTANEOUS ABSORPTION OF C^{14}-LABELED SALICYLIC ACID

EFFECT OF REDUCED CONCENTRATION (See Legend)

Skin Depth from Surface in Microns(μ)	DPM in 100 Micron Sections of Skin Replicates						Average DPM
	#1	#2	#3	#4	#5	#6	
100	63,913	178,802	66,986	58,802	101,912	47,265	86,280
200	4,457	4,912	4,500	4,013	5,501	4,645	4,671
300	3,171	4,128	4,235	3,912	4,234	4,471	4,025
400	3,592	3,618	3,354	2,913	3,850	3,398	3,454
500	3,036	3,147	2,549	2,930	3,094	3,726	3,080
600	2,593	2,462	1,980	2,281	2,298	3,012	2,438
Total Activity*	16,849	18,267	16,618	16,049	18,977	19,252	

AVERAGE TOTAL ACTIVITY* = 17,669 ± 1,340**

Legend

1. Drug vehicle - Hydrophilic Oint. USP XVII
2. Rubbing time - 10 seconds
3. Rest time - 240 minutes
4. Skin sample diameter - 8 mm
5. Skin type - bald
6. Direction of slicing - subcut. to epid.
7. Drug concentration in vehicle - 5%
8. Specific activity of drug in vehicle - 45 μc/Gm

* Excludes first 100 μ which contain contamination from surface

** Standard Deviation

TABLE XIX

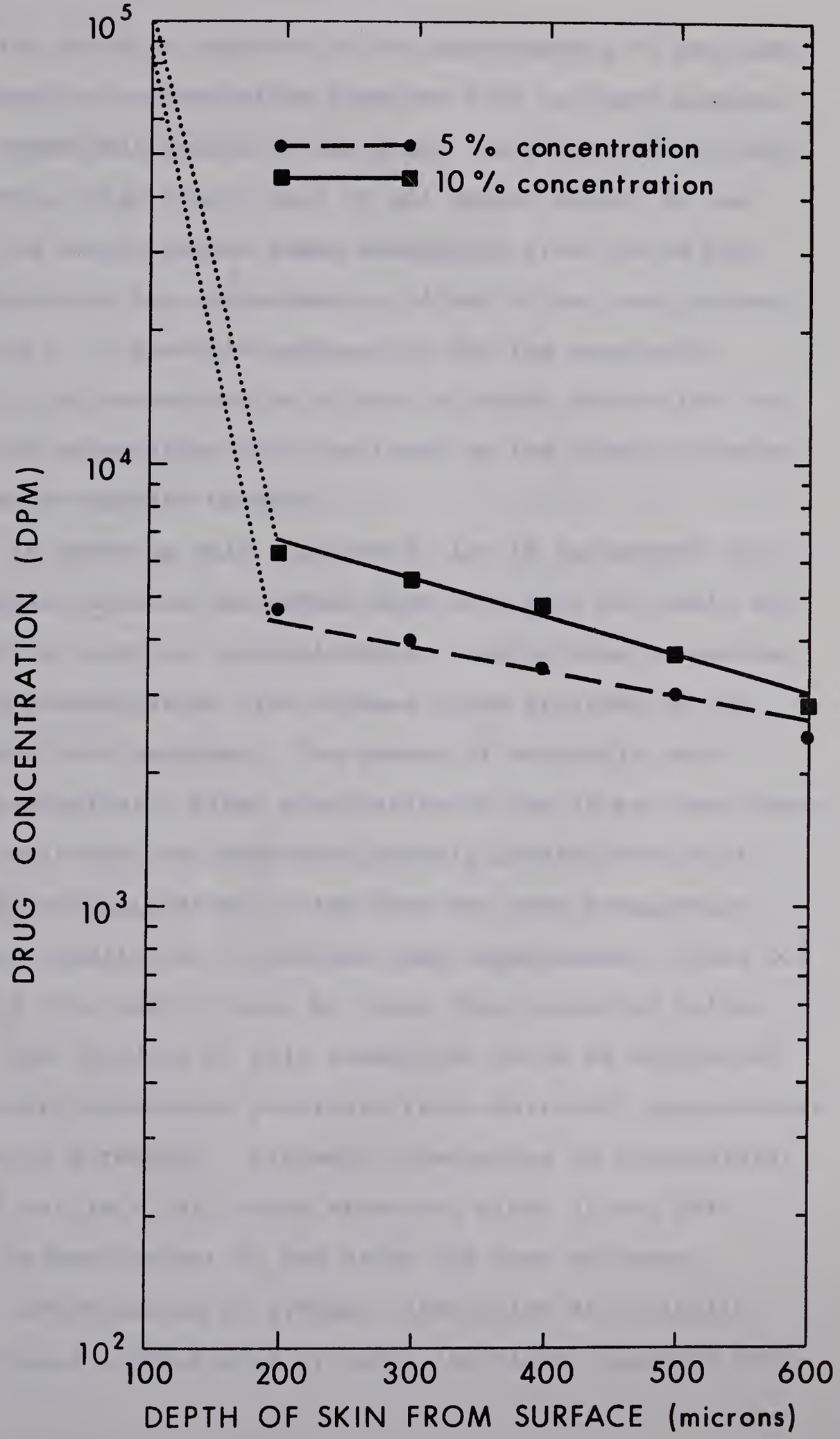
COMPARISON OF THEORETICAL (Eq. 8) AND EXPERIMENTAL ABSORPTION (DPM) OF 5 AND 10% C^{14}-LABELED SALICYLIC ACID BY SKIN AND PLASMA

Concentration (%)	ABSORPTION (DPM)			
	SKIN		PLASMA	
	Theor.	Exptl.	Theor.	Exptl.
5*	17,669	17,669	1,191	1,191
10	24,780	23,348	1,670	3,115

* Theoretical absorption at this concentration was arbitrarily taken to be the same as that obtained experimentally.

Experimental absorption by the skin increased by approximately 32 per cent upon doubling the concentration of salicylic acid applied. This satisfactory agreement with the 40 per cent increase predicted by equation 8 indicates that the method developed for measuring intracutaneous absorption provides a reasonably accurate measurement of the effect which concentration was expected to have upon drug absorption from a suspension system. An explanation for the fact that the experimental results were slightly lower than those predicted may be found by comparing the absorption gradients of the two preparations (Figure 19). If absorption from both the five and 10 per cent preparations varied directly with concentration as predicted by equation 5, the slopes of the two gradients would be expected to be identical. On this basis, the concentration of drug absorbed from the 10 per cent

Figure 19: Concentration gradients of C^{14}-labeled salicylic acid in the skin absorbed from preparations of different drug concentrations.


5 % concentration
10 % concentration
DRUG CONCENTRATION (DPM)
10^5
10^4
10^3
10^2
100
200
300
400
500
600
DEPTH OF SKIN FROM SURFACE (microns)

preparation would be expected to be approximately 40 per cent higher than the concentration from the five per cent preparation at comparable depths of the skin. As demonstrated, this relationship holds fairly well at all depths except at the 500 and 600 micron levels where absorption from the 10 per cent preparation was approximately 24 and 18 per cent higher respectively. A possible explanation for the apparently abnormally low concentration of drug at these depths for the 10 per cent preparation could be based on the close proximity of the major vascular network.

In pursuing this hypothesis, let it be assumed that this vascular network can remove from this area virtually all drug above a specific concentration. Let it also be assumed that this concentration lies between those provided by the two preparations employed. The amount of salicylic acid removed systemically after application of the 10 per cent preparation would then be disproportionately greater than that removed after application of the five per cent preparation. Under such conditions, it follows that experimental values for tissues at this depth should be lower than predicted values.

The validity of this assumption could be determined by developing absorption gradients from additional preparations of differing strengths. Although interesting to contemplate, this work was left for future attention since it was felt that the primary object of the study had been achieved.

Determination of systemic absorption of salicylic acid was again accomplished by using the plasma analysis tech-

nique. Values obtained for both the five and the 10 per cent preparations are presented in Table XX. A comparison of the experimental values and those derived on a theoretical basis is shown in Table XIX.

From the information so presented, it can be seen that the systemic absorption of salicylic acid increased approximately 162 per cent through doubling the concentration of the drug preparation. This increase did not agree well with the predicted and experimental intracutaneous absorption increases of 40 and 32 per cent respectively. It appeared that removal of drug from the deeper tissues by the rete cutaneum as suggested above, was in fact much greater from the 10 per cent preparation than from the five per cent counterpart.

For this reason it was concluded that, within the parameters of the present study, equation 5 is not suitable for use in predicting the effect of concentration on systemic absorption. The values also indicate that the effect of concentration on percutaneous absorption should be measured for the intracutaneous and systemic phases individually.

TABLE XX

SYSTEMIC ABSORPTION OF C^{14}-LABELED SALICYLIC ACID (See Legend)

EFFECT OF CONCENTRATION

Concentration (%)	Replicate Numbers	Average DPM[a] in 0.5 ml Plasma Replicates #1	#2	#3	#4	#5	#6
5	DPM	1,291	1,210	1,740	528	1,134	1,268
	Correction Factor[b]	0.96	0.98	1.03	1.11	0.98	0.97
	Corrected DPM	1,239	1,186	1,792	586	1,111	1,230
	AVERAGE DPM AMONG REPLICATES = 1,191 ± 384[c]						
10	DPM	1,758	2,326	2,784	4,238	3,636	4,136
	Correction Factor[b]	1.08	1.04	0.99	0.97	0.95	0.98
	Corrected DPM	1,899	2,419	2,756	4,111	3,454	4,053
	AVERAGE DPM AMONG REPLICATES = 3,115 ± 903[c]						

Legend:

1. Drug vehicle - Hydrophilic Oint. USP XVII
2. Specific activity - 5% = 46 μc/Gm
   10% = 90 μc/Gm.
3. Rubbing time - 10 secs.
4. Rest time - 240 mins.

a. Average of duplicate determinations for each replicate.
b. Corrected to constant plasma volume (see p. 58).
c. Standard Deviation.

SUMMARY

A method for quantitatively measuring intracutaneous absorption has been presented. The method involved the use of guinea pig skin to measure the transepidermal absorption of C^{14}-labeled salicylic acid. A number of variables were found to influence the uniform application of drug when distributed in a viscous vehicle to a specific surface area. The procedures used to identify and control these factors and the procedures used to make possible a quantitative recovery of absorbed drug have been described.

The data obtained from this work indicated that the intracutaneous absorption of salicylic acid did not appear to change with different rubbing times. On the otherhand, the systemic absorption of the drug did appear to change under these conditions. There was also some evidence to suggest that the nature of the vehicle in which the salicylic acid was presented had a measurable effect on the subsequent absorption of the drug.

The intracutaneous and systemic absorptions of salicylic acid were measured as a function of rest time. The results showed that the rates of absorption by both phases changed with time and that such rates differed for the two phases under the conditions of the investigation. These results suggested that intracutaneous and systemic absorption should be measured individually and that absorption be studied at more than one rest time.

The effect of changing the concentration of applied

drug was evaluated to determine the ability of the developed method to measure the predicted effect of such a variable on intracutaneous absorption. The experimental data obtained compared favorably with those derived theoretically and thus supported the value of the method developed as a quantitative measure of intracutaneous absorption. The experimental values for systemic absorption, however, did not agree with those predicted nor with the experimental intracutaneous results. This further supported the importance of measuring absorption by the two phases individually.

The experiments on the percutaneous absorption of salicylic acid as a function of rest time and of concentration were not intended to, and did not provide an exhaustive evaluation of the effect of these variables upon absorption. These experiments did, however, appear to satisfy the objective of developing a quantitative method for measuring drug absorption by the skin tissues and to substantiate the importance of measuring such absorption as well as systemic absorption.

CONCLUSIONS

1. A method using guinea pig skin has been developed for quantitatively measuring the intracutaneous absorption of radioisotope-labeled compounds by the transepidermal route.

2. The relative distribution of absorbed drug at different depths of the dermis is also determined by the method described.

3. It has been demonstrated that systemic and intracutaneous absorption of C^{14}-labeled salicylic acid are not proportional when taken as functions of time and of concentration.

4. The intracutaneous absorption values of salicylic acid measured experimentally agreed in most cases with absorption predicted on a theoretical basis for both rest time and concentration studies.

5. Experimental results for systemic absorption did not agree with those predicted on the basis of theoretical considerations.

6. Changes in the rates of intracutaneous and systemic absorption of salicylic acid indicate that the effects of variables such as concentration on percutaneous absorption should be evaluated at more than one rest time.

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